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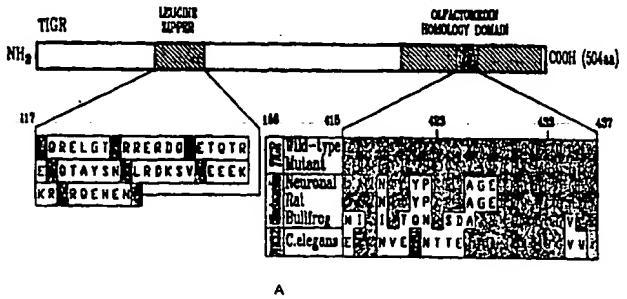
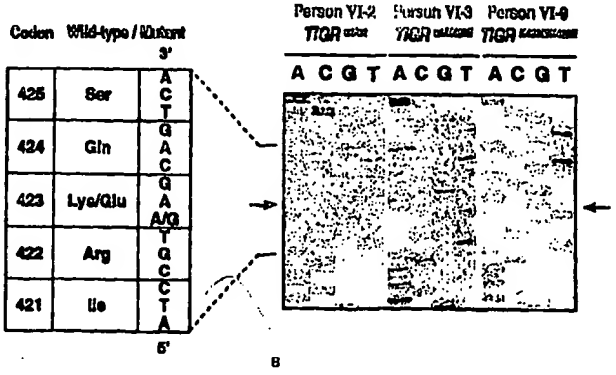
(57) Abstract

The present invention relates to the surprising discovery that in an autosomally inherited disease, a homozygote mutant is found to be phenotypically normal and to the uses of such a discovery. The present invention further relates to easy and efficient means to detect mutations in the *TIGR/MYOC* gene. Further, the present invention relates to methods to diagnose and treat glaucoma or other diseases or conditions in which homoallelic complementation is observed.





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TITLE OF THE INVENTION

MOLECULAR DIAGNOSTIC OF GLAUCOMAS ASSOCIATED WITH CHROMOSOME 1, AND METHOD OF TREATMENT THEREOF

FIELD OF THE INVENTION

The present invention relates to the identification of mutations in the *TIGR/MYOC* gene in the *GLCIA* locus and the detection of these mutations in individuals. The invention also relates to the identification of individuals who are genotypically homozygote mutants of an autosomal dominant inherited disease and yet display a normal phenotype.

BACKGROUND OF THE INVENTION

Glaucoma encompasses a complex of ocular-disease entities characterized by an optic neuropathy in which degeneration of retinal ganglion cells leads to a characteristic excavation of the head of the optic nerve (Shields et al., 1996, The Glaucomas, 2:717-725). Such damage causes progressive narrowing of the visual fields and, when uncontrolled, blindness. Affected people often have ocular hypertension defined as intraocular pressures consistently >21 mm Hg in both eyes. Although ocular hypertension is no longer an obligatory diagnostic criterion for glaucoma, it is still recognized as one of the most important risk factors (Wilson et al., 1996, The Glaucomas, 2:753-763). Until now, a diagnosis of glaucoma is made after observation of the characteristic atrophy of the optic nerve, which is associated with typical visual field defects.

In 1992, the World Health Organization estimated that, in the global population, 5.2 million people were blind as a result of glaucoma (Thylefors et al., 1994, World Health Organ. Bull., 72:323-326), making it the third leading cause of blindness worldwide. The most common form is adult-onset primary open-angle glaucoma (MIM 137760; McKusick, 1994, Johns Hopkins University Press, p. 272), which represents ~50% of all cases of

glaucoma. Among Caucasians, this form of the disorder affects ~2% of the population >45 years old (Leske, 1983, Am. Epidemiol., 118:166-191; Thylefors et al., 1994, supra; Wilson et al., 1996, supra). In African Americans, prevalence of adult-onset open-angle glaucoma is three to four times higher than that observed in White Americans. More than 5 million North Americans may have some form of glaucoma, but at least half of them may not be aware of it.

The glaucomas traditionally have been grouped into three categories: open angle, closed angle (also termed "angle closure"), and congenital. Each subtype has been further arbitrarily subdivided into *primary*, when the anterior chamber of the eye appears normal and no cause for glaucoma can be identified, or *secondary*, when glaucomas are caused by underlying ocular or systemic conditions (Shields et al., 1996, supra). Whereas the division between open and closed angles refers to the configuration of the irido-corneal angle in the anterior chamber of the eye, congenital glaucoma is used to define one of the many types of developmental glaucoma that usually occurs within the 1st year of life. The majority (60%-70%) of primary glaucomas are of the open-angle type. Primary open-angle glaucomas have been further subdivided into two groups according to age at onset, severity, and mode of inheritance: the more prevalent is middle- to late-age-onset chronic open-angle glaucoma (COAG), by convention diagnosed after age 35 years and characterized by its slow, insidious course (Shields et al., 1996, supra; Wilson et al., 1996, supra). The less common form, juvenile open-angle glaucoma (JOAG), occurs between 3 years of age and early adulthood and generally manifests highly elevated intraocular pressures with no angle abnormalities (Goldwyn et al., 1970, Arch. Ophtalmol., 84:579-582; François, 1980, Am. J. Ophtalmol., 3:429-449; Johnson et al., 1996*a*, The Glaucomas, 1:39-54).

Although the precise molecular defects leading to open-angle glaucomas remain partly unknown, numerous advances in basic and clinical sciences have begun to identify the molecular basis of glaucomas by mapping the gene loci involved in the disease process. Due to recent mapping

successes, the different forms of glaucoma will be further identified by the names of the loci to which they have been localized. According to the Human Genome Organization/Genome Database nomenclature, "*GLC*" is the general symbol for the glaucoma genes; "1", "2", and "3" are, respectively, the symbols for the open-angle, angle-closure, and congenital subtypes of glaucoma; and "A", "B", and "C" refer, respectively, to the first, second, or third gene mapped in each subgroup.

JOAG is a rare but aggressive form of glaucoma that usually segregates in an autosomal dominant fashion with high penetrance (Stokes, 1940, Arch. Ophthalmol., 24:885-909; Crombie et al., 1964, Br. J. Ophtalmol., 48:143-147; Lee et al., 1985, Ann. Ophtalmol., 17:739-741; Johnson et al., 1993, Ophthalmology, 100:524-529). In a single large American pedigree affected by an autosomal dominant form of JOAG, Sheffield et al. (1993, Nat. Genet., 4:47-50) located a gene responsible for this condition, at 1q21-q31. This locus, being the first open-angle glaucoma locus to be mapped, was named "*GLC1A*." The *TIGR/MYOC* glaucoma disease gene consistently was first associated with onset of the glaucoma phenotype before the age of 45 years, highly elevated intraocular pressures, and typical excavation of the head of the optic nerve. Gonioscopy showed open angles with no anterior-chamber abnormalities. The *GLC1A* locus has subsequently been reported by Nguyen et al. in US Patent 5,606,043 to encode the trabecular meshwork induced glucocorticoid response (*TIGR*) gene. The gene sequence was first submitted (13-JAN-1997) by Nguyen et al. to the GeneBank accession # U85257. The *TIGR* sequence was modified on 19 April 1997 in GeneBank following modifications by Nguyen submitted on 02-APR-1997. The accession number stayed the same # U85257.

Genetic maps of the human genome can be exploited to rapidly locate human monogenic disorders. The final version of the Généthon linkage map, which spans close to 100 % of the human genome, was published in March 1996 (Dib et al., 1996, Nature, 380:152-154). This map consists of

5,264 short tandem (AC/TG)n repeat polymorphisms with a mean heterozygosity of 70%.

The nomenclature system for the markers is well known in the field. The nomenclature used is decided by the Human Genome Organization (HUGO) nomenclature committee. It is as follows: for anonymous DNA sequences, the convention is to use D which is equivalent to DNA followed by 1-22, X or Y to denote the chromosomal number and location, then S stands for a unique segment and finally a serial number. For example, marker D2S2161 is a DNA marker located on chromosome 2 representing a unique segment. Its serial number is 2161.

The nomenclature for the glaucoma genes is the following: "*GLC*" is the general symbol for the glaucoma genes; "1", "2", and "3" are, respectively, the symbols for the open-angle, angle-closure, and congenital subtypes of glaucoma; and, "A", "B" and "C" refer, respectively, to the first, second, or third gene mapped in each subgroup. For example, the *GLC1A* locus was the first open-angle glaucoma locus to be mapped, in this case to chromosome 1q23-q25 in 1993. It was later identified as the trabecular meshwork inducible glucocorticoid response gene product (*TIGR*) (Stone et al, 1997, Science, 275: 668-670). The *TIGR* gene is also known as *MYOC*.

These markers are accessible to all individuals. The central data resource for the human gene mapping effort is the Genome Data Base (GDB). It was established at Johns Hopkins University, School of Medicine. GDB is updated regularly. It collects, organizes, stores and distributes human genome mapping information. GDB is accessible electronically at WWW-URL: http://gdbwww.gdb.org/.

Alternatively, all the markers disclosed herein, except D6S967, are short (CA)n repeat markers that have been developed in the Généthon laboratory near Paris, France. These markers are also accessible electronically at WWW-URL: http://www.genethon.fr/.

Therefore markers are accessible either at GDB or at Généthon.

The first mutations identified in the *TIGR* gene that have been shown to give rise to glaucoma were first reported by Stone et. al (Science, 1997, 275:668-670). Twenty-eight mutations have now been reported. The methodology used to identify these mutations was by amplifying overlapping regions by polymerase chain reaction (PCR), performing single-strand conformational polymorphism (SSCP) on the amplification products and sequencing those DNA products that produced aberrant band pattern on the SSCP. No quick method for mutational analyses for the *TIGR* has been proposed.

The prior art as a whole teaches that a homozygote mutant for an autosomal dominant disease should display a higher penetrance and severity for a human disorder than a heterozygote mutant. Heterozygotes for an autosomal dominant disease often exhibit variable penetrance.

The present description refers to a number of documents the content of which is herein incorporated by reference.

SUMMARY OF THE INVENTION

The invention concerns the mutational analyses in the *GLC1A* gene locus encoding the *TIGR* gene (GeneBank accession no. U85257; SEQ ID NO:1).

The present invention provides means to identify at least two nucleotide changes in the DNA sequence coding for *TIGR* that result in an amino acid change in the *TIGR* gene.

The invention further demonstrates that these amino acid changes result in mutations producing a disease state in individuals, the disease being glaucoma.

The early detection of individuals at risk for developing glaucoma is an important aspect of this invention. Early detection allows for

intervention prior to the genesis of the disease process and disease progression and may obviate the symptoms and the onset of the disease.

A method for mutation analyses called amplification refractory mutation system (ARMS), that is simple, quick and adapted to glaucoma is disclosed herein. The proposed invention relates to the inclusion of primers and probes for the amplification and detection of all mutations in the *TIGR* gene. Although the invention teaches the use of the method of ARMS for glaucoma, the invention is not so limited. Other methods of mutation analyses known in the field, such as allele specific oligonucleotide (ASO), denaturing gradient gel electrophoresis (DGGE) and artificially created restriction site (ACRS), can also be used.

The mutation detection and analyses thereof can be performed on either genomic DNA or cDNA by any method known to a person skilled in the art.

In addition the applicant has demonstrated for the first time a new type of dominance in mammals in which heterozygotes have a much higher penetrance rate for a disease gene mutation than their homozygotic counterparts.

Further, the present invention provides for the first time the identification in an autosomal dominant disease, of a homozygote mutant which is phenotypically normal, even though such an individual may give rise to an affected heterozygote offspring. This homoallelic complementation phenomenon has now been observed with 4 different patients.

The invention provides applications and uses for such a discovery. These include but are not limited to:

a) treatment of a heterozygote mutant (affected individuals with overexpressed mutant protein to induce protein complementation such that normal protein function can be restored, this application will apply to any autosomal dominant disease exhibiting the same mode of action as described herein (i.e. homoallelic complementation).

b) similarly an individual being a heterozygote for an autosomal dominant disorder exhibiting the same mode of action as described herein can be treated by gene therapy, such that a mutant allele is inserted into a vector and delivered to an individual thereby negating the effect of the heterozygote mutation by either allelic or protein complementation.

c) with the new knowledge of the present invention, a transgenic animal designed to carry a deleterious autosomal dominant mutation can be used to assess the requirement to produce a phenotypically normal animal, by either allelic complementation or protein complementation.

d) a diagnostic means to identify phenotypically normal genotypically mutant individuals that can transmit the mutant allele to their offsprings.

e) the teachings of the present invention can be used for showing dimezisation of *TIGR* peptides.

The present invention therefore also provides the means to identify novel mutations in the *TIGR* gene, wherein these mutations give rise to glaucoma. These mutations can also be identified by any other means known to a person skilled in the art. As well, the diagnostic methods of the present invention can be adapted in a kit format comprising probes, primers, oligonucleotides and reagents commonly known in the art to provide the means for detecting mutations that may cause glaucoma. The present invention further provides methods to treat diseases or conditions associated with homoallelic complementation.

The mutation analysis according to the present invention is useful for screening individuals at risk for glaucoma. Such individuals may have a family history of glaucoma, and for identifying individuals carrying a mutation in the glaucoma gene enabling early treatment which may obviate or minimise the progression of the disease.

In another embodiment of the present invention, there is provided a kit comprising all the necessary reagents to carry out the herein

described methods of detection. As known to the person of ordinary skill, the kit comprises container means comprising oligonucleotide sequences or antibodies (or binding proteins) and reagents such as washing reagents, reagents for detection purposes and the like. It will also be readily recognized that the nucleic acid sequences or antibodies of the present invention could be incorporated into established kit formats.

The present invention thus discloses, for the first time, a mechanism termed "homoallelic complementation" wherein for example the K423E mutation is acting in a dominant negative fashion, thereby resulting in a defective *TIGRwt/K423E* protein heteromaltemers but functional *TIGR K23E/K23E* homopolymers. This form of interaction may be interpreted as being somewhat similar to the "metabolic interference model" that suggested a deleterious defect due to interference between the protein products of the two different alleles (Johnson, 1980, Am. J. Hum. Genet. 32:374-86). The homoallelic complementation model of the present invention, however, further proposes that functional *TIGRwt/wt* and/or *TIGR K23E/K23E* homopolymers are generated by an admixture of normal and mutant subunits in *TIGRwt/K432E* heterozygotes, thereby explaining, at least in part, the phenotypic variability observed in effected carriers as well as the unaffected mutant homozygotes described herein. It should be noted that Crick et al. (1964, J. Molec. Biol. 8:161-165) had previously suggested the theory of inter-allelic complementation.

While homoallelic complementation has been identified and validated with glaucoma, it is suspected to be a phenomenon which is not limited thereto. For example, a form of epilepsy and mental retardation linked to chromosome X, which only affects women, has been reported (Ryan et al., 1997, Nature Genetics 17:92-95).

In accordance with the present invention, there is thus provided an isolated DNA comprising the nucleotide sequence defined in SEQ. ID. NO.: 1, wherein a mutation is identified therein.

There is also provided a method for detecting a mutant allele of the *TIGR/MYOC* gene which comprises the steps of contacting a DNA sample taken from an individual with an oligonucleotide as defined in claims 3, 5 or 7 and with an oligonucleotide primer of the present invention; obtaining an amplified product in an amplification reaction; and detecting the amplification product as an indication of the presence of said mutant allele.

In accordance with the present invention, there is also provided a method for detecting a non-mutant allele of the *TIGR/MYOC* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide primer (or a probe enabling a distinction between wild type and mutant); obtaining an amplified product in an amplification reaction; and detecting the amplification product as an indication of the presence of the non-mutant allele.

The application further relates to a kit for the detection of mutations in the *TIGR* gene comprising an oligonucleotide of the present invention; and suitable reagents required for obtaining amplified products in an amplification reaction.

In addition, in accordance with the present invention, there is provided a method to counteract glaucoma in a heterozygotic carrier of TIGR mutations, an overexpression of mutated TIGR protein in a patient, thereby rendering the phenotype of said patient normal by homoallelic complementation.

Further, in accordance with the present invention, there is provided a method to counteract and/or treat heterozygotic carriers of an autosomal dominant inherited disorder caused by a protein that forms homomultimers, comprising at least one of an overexpression of the mutated protein and an inhibition of the normal protein in a patient, thereby rendering said phenotype normal by homoallelic complementation.

Further, in accordance with the present invention, there is provided a method to counteract a disease phenotype in a patient, wherein the

disease phenotype is associated with the presence of a heterozygotic mutation in a protein in the patient, the method comprising at least one of an elevation of the expression of a mutated protein and an inhibition of expression and/or activity of the normal protein, to counteract the affected phenotype, thereby rendering the phenotype normal by one of homoallelic complementation and haploinsufficiency.

DEFINITIONS AND TECHNOLOGICAL BACKGROUND

Nucleotide sequences are presented herein by single strand, in the 5' to 3' direction, from left to right, using the one letter nucleotide symbols as commonly used in the art and in accordance with the recommendations of the IUPAC-IUB Biochemical Nomenclature Commission.

Unless defined otherwise, the scientific and technological terms and nomenclature used herein have the same meaning as commonly understood by a person of ordinary skill to which this invention pertains. Generally, the procedures for cell cultures, infection, molecular biology methods and the like are common methods used in the art. Such standard techniques can be found in reference manuals such as for example Sambrook et al. (1989, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratories) and Ausubel et al. (1994, Current Protocols in Molecular Biology, Wiley, New York).

The present description refers to a number of routinely used recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected examples of such rDNA terms are provided for clarity and consistency.

As used herein, "*TIGR*" refers to the trabecular meshwork inducible glucocorticoid response gene product, also known as *MYOC*, present at the *GLC1A* locus. Herein, "*TIGR*" therefore refers to *TIGR/MYOC*. It should be noted that the *TIGR/MYOC* gene is sometimes referred to as the *GLC1A* gene.

As used herein, the terminology "homoallelic complementation" refers to the novel description of mutant homozygotes being

phenotypically normal with respect to an autosomal dominant inherited disease. It should be noted that although the present invention focuses on autosomal dominant inherited diseases, the present invention also applies to haploinsufficiency. Broadly stated, the mechanism would be similar to a suggested locus with a wild-type allele A and a mutant allele A', such that homozygosity for either allele, has no phenotypic consequences, but the heterozygous state AA' leads to a deleterious defect due to interference between the protein products of the two different alleles.

As used herein, "nucleic acid molecule", refers to a polymer of nucleotides. Non-limiting examples thereof include DNA (i.e. genomic DNA, cDNA) and RNA molecules (i.e. mRNA). The nucleic acid molecule can be obtained by cloning techniques or synthesized. DNA can be double-stranded or single stranded (coding strand or non-coding strand [antisense]).

The term "recombinant DNA" as known in the art refers to a DNA molecule resulting from the joining of DNA segments. This is often referred to as genetic engineering.

The term "DNA segment", is used herein, to refer to a DNA molecule comprising a linear stretch or sequence of nucleotides. This sequence when read in accordance with the genetic code, can encode a linear stretch or sequence of amino acids which can be referred to as a polypeptide, protein, protein fragment and the like.

The terminology "amplification pair" or "primer pair" refers herein to a pair of oligonucleotides (oligos) of the present invention, which are selected to be used together in amplifying a selected nucleic acid sequence by one of a number of types of amplification processes, preferably a polymerase chain reaction. Other types of amplification processes include ligase chain reaction, strand displacement amplification, or nucleic acid sequence-based amplification, as explained in greater detail below. As commonly known in the art, the oligos are designed to bind to a complementary sequence under selected conditions.

It will be clear to the person of ordinary skill that the present invention can be adapted to the detection of numerous mutations in *TIGR/MYOC*. As exemplified hereinbelow, using a particular embodiment of the present invention using ARMS with oligos having the sequences of SEQ ID NO:5 and SEQ ID NO:7, mutations *His366Gln* and *LYS424GLn* were identified.

The nucleic acid (i.e. DNA or RNA) for practising the present invention may be obtained according to well known methods.

Oligonucleotide probes or primers of the present invention may be of any suitable length, depending on the particular assay format and the particular needs and targeted genomes employed. In general, the oligonucleotide probes or primers are at least 10 nucleotides in length, preferably between 15 and 24 nucleotides, and they may be adapted to be especially suited to a chosen nucleic acid amplification system. As commonly known in the art, the oligonucleotide probes and primers can be designed by taking into consideration the melting point of hydrizidation thereof with its targeted sequence (in Sambrook et al., 1989, Molecular Cloning - A Laboratory Manual, 2nd Edition, CSH Laboratories; Ausubel et al., 1989, in Current Protocols in Molecular Biology, John Wiley & Sons Inc., N.Y.).

"Nucleic acid hybridization" refers generally to the hybridization of two single-stranded nucleic acid molecules having complementary base sequences, which under appropriate conditions will form a thermodynamically favored double-stranded structure. Examples of hybridization conditions can be found in the two laboratory manuals referred above (Sambrook et al., 1989, *supra* and Ausubel et al., 1989 supra) and are commonly known in the art. In the case of a hybridization to a nitrocellulose filter, as for example in the well known Southern blotting procedure, a nitrocellulose filter can be incubated overnight at 65°C with a labeled probe in a solution containing 50% formamide, high salt (5 x SSC or 5 x SSPE), 5 x Denhardt's solution, 1% SDS, and 100 µg/ml denatured carried DNA (i.e. salmon sperm DNA). The non-specifically binding probe can then be washed off

the filter by several washes in 0.2 x SSC/0.1% SDS at a temperature which is selected in view of the desired stringency: room temperature (low stringency), 42°C (moderate stringency) or 65°C (high stringency). The selected temperature is based on the melting temperature (Tm) of the DNA hybrid. Of course, RNA-DNA hybrids can also be formed and detected. In such cases, the conditions of hybridization and washing can be adapted according to well known methods by the person of ordinary skill. High stringency conditions will be preferably used (Sambrook et al.,1989, supra).

Probes or nucleic acid molecules of the invention can be utilized with naturally occurring sugar-phosphate backbones as well as modified backbones including phosphorothioates, dithionates, alkyl phosphonates and α-nucleotides and the like. Modified sugar-phosphate backbones are generally taught by Miller, 1988, Ann. Reports Med. Chem. 23:295 and Moran et al., 1987, Nucleic acid molecule. Acids Res., 14:5019. Probes of the invention can be constructed of either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), and preferably of DNA.

The types of detection methods in which probes can be used include Southern blots (DNA detection), dot or slot blots (DNA, RNA), and Northern blots (RNA detection). Although less preferred, labelled proteins could also be used to detect a particular nucleic acid sequence to which it binds. Other detection methods include kits containing probes on a dipstick setup and the like.

Although the present invention is not specifically dependent on the use of a label for the detection of a particular nucleic acid sequence, such a label might be beneficial, by increasing the sensitivity of the detection. Furthermore, it enables automation. Probes can be labelled according to numerous well known methods (Sambrook et al., 1989, supra). Non-limiting examples of labels include ^{3}H, ^{14}C, ^{32}P, and ^{35}S. Non-limiting examples of detectable markers include ligands, fluorophores, chemiluminescent agents, enzymes, and antibodies. Other detectable markers for use with probes, which

can enable an increase in sensitivity of the method of the invention, include biotin and radionucleotides. It will become evident to the person of ordinary skill that the choice of a particular label dictates the manner in which it is bound to the probe.

As commonly known, radioactive nucleotides can be incorporated into probes of the invention by several methods. Non-limiting examples thereof include kinasing the 5' ends of the probes using gamma ^{32}P ATP and polynucleotide kinase, using the Klenow fragment of Pol I of *E. coli* in the presence of radioactive dNTP (i.e. uniformly labelled DNA probe using random oligonucleotide primers in low-melt gels), using the SP6/T7 system to transcribe a DNA segment in the presence of one or more radioactive NTP, and the like.

As used herein, "oligonucleotides" or "oligos" define a molecule having two or more nucleotides (ribo or deoxyribonucleotides). The size of the oligo will be dictated by the particular situation and ultimately by the particular use thereof, and adapted accordingly by the person of ordinary skill. An oligonucleotide can be synthetised chemically or derived by cloning according to well known methods.

As used herein, a "primer" defines an oligonucleotide which is capable of annealing to a target sequence, thereby creating a double stranded region which can serve as an initiation point for DNA synthesis under suitable conditions.

Amplification of a selected, or target, nucleic acid sequence may be carried out by a number of suitable methods. See generally Kwoh et al., 1990, (Am. Biotechnol. Lab. 8:14-25). Numerous amplification techniques have been described and can be readily adapted to suit the particular needs of a person of ordinary skill. Non-limiting examples of amplification techniques include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), transcription-based amplification, the Qβ replicase system and NASBA (Kwoh et al., 1989, Proc. Natl. Acad. Sci. USA 86,

1173-1177; Lizardi et al., 1988, BioTechnology 6:1197-1202; Malek et al., 1994, Methods Mol. Biol., 28:253-260; and Sambrook et al., 1989, *supra*). Preferably, amplification will be carried out using PCR.

Polymerase chain reaction (PCR) is carried out in accordance with known techniques. See, e.g., U.S. Pat. Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188 (the disclosures of all three U.S. Patent are incorporated herein by reference). In general, PCR involves, a treatment of a nucleic acid sample (e.g., in the presence of a heat stable DNA polymerase) under hybridizing conditions, with one oligonucleotide primer for each strand of the specific sequence to be detected. An extension product of each primer which is synthesized is complementary to each of the two nucleic acid strands, with the primers sufficiently complementary to each strand of the specific sequence to hybridize therewith. The extension product synthesized from each primer can also serve as a template for further synthesis of extension products using the same primers. Following a sufficient number of rounds of synthesis of extension products, the sample is analysed to assess whether the sequence or sequences to be detected are present. Detection of the amplified sequence may be carried out by visualization following EtBr staining of the DNA following gel electrophoresis, or using a detectable label in accordance with known techniques, and the like. For a review on PCR techniques (see PCR Protocols, A Guide to Methods and Amplifications, Michael et al., Eds, Acad. Press, 1990).

Ligase chain reaction (LCR) is carried out in accordance with known techniques (Weiss, 1991, Science 254:1292). Adaptation of the protocol to meet the desired needs can be carried out by a person of ordinary skill. Strand displacement amplification (SDA) is also carried out in accordance with known techniques or adaptations thereof to meet the particular needs (Walker et al., 1992, Proc. Natl. Acad. Sci. USA 89:392-396; and ibid., 1992, Nucleic Acids Res. 20:1691-1696).

As used herein, the term "gene" is well known in the art and relates to a nucleic acid sequence defining a single protein or polypeptide. A

"structural gene" defines a DNA sequence which is transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise the a specific polypeptide or protein. It will be readily recognized by the person of ordinary skill, that the nucleic acid sequences of the present invention can be incorporated into anyone of numerous established kit formats which are well known in the art.

The term "vector" is commonly known in the art and defines a plasmid DNA, phage DNA, viral DNA and the like, which can serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the art.

The term "expression" defines the process by which a structural gene is transcribed into mRNA (transcription), the mRNA is then being translated (translation) into one polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle, as described above, but designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene (inserted sequence) is usually placed under the control of control element sequences such as promoter sequences. The placing of a cloned gene under such control sequences is often referred to as being "operably linked" to control elements or sequences.

Expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

As used herein, the designation "functional derivative" denotes, in the context of a functional derivative of a sequence, whether nucleic acid or amino acid sequence, a molecule that retains a biological activity (either functional or structural) that is substantially similar to that of the original sequence. This functional derivative or equivalent may be a natural derivative

or may be prepared synthetically. Such derivatives include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided that the biological activity of the protein is conserved. The same applies to derivatives of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained. When relating to a protein sequence, the substituting amino acid has chemico-physical properties which are similar to that of the substituted amino acid. The similar chemico-physical properties include, similarities in charge, bulkiness, hydrophobicity, hydrophylicity and the like. The term "functional derivatives" is intended to include "fragments", "segments", "variants", "analogs" or "chemical derivatives" of the subject matter of the present invention.

Thus, the term "variant" refers herein to a protein or nucleic acid molecule which is substantially similar in structure and biological activity to the protein or nucleic acid of the present invention.

The functional derivatives of the present invention can be synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

As used herein, "chemical derivatives" is meant to cover additional chemical moieties not normally part of the subject matter of the invention. Such moieties could affect the physico-chemical characteristic of the derivative (i.e. solubility, absorption, half life and the like, decrease of toxicity). Such moieties are exemplified in Remington's Pharmaceutical Sciences (1980). Methods of coupling these chemical-physical moieties to a polypeptide are well known in the art.

The term "allele" defines an alternative form of a gene which occupies a given locus on a chromosome.

As commonly known, a "mutation" is a detectable change in the genetic material which can be transmitted to a daughter cell. As well known, a mutation can be, for example, a detectable change in one or more

deoxyribonucleotide. For example, nucleotides can be added, deleted, substituted for, inverted, or transposed to a new position. Spontaneous mutations and experimentally induced mutations exist. The result of a mutations of nucleic acid molecule is a mutant nucleic acid molecule. A mutant polypeptide can be encoded from this mutant nucleic acid molecule.

As used herein, the term "purified" refers to a molecule having been separated from a cellular component. Thus, for example, a "purified protein" has been purified to a level not found in nature. A "substantially pure" molecule is a molecule that is lacking in all other cellular components.

The term "autosome" defines any chromosome other that the sex chromosomes, X and Y.

The term "dominant" refers to an allele that determines the phenotype displayed in a heterozygote with another normal or mutant allele.

The terminology "transgenic animal" defines an animal that has had its germ line genetically modified to give rise to a progeny animal that is different from the parental type and carrying the modification in its germ line.

Non-human transgenic animals of the invention comprise animals having transgenic alteration of an endogenous gene which shows homoallelic complementation, in accordance with the present invention. Such non-human animals are commonly known in the art and include vertebrates, and more especially mammalians, and especially rodents such as rats and more particularly mice. These transgenic animals have had introduced into their genomes, by non-natural means (i.e. by manipulation), one or more gene which does not occur naturally in the animal. These non-naturally occuring genes in the animal (known as transgenes) may be from the same species as the animal, although in such a case, the gene or genes are in a different configuration and/or chromosomal location. A transgenic non-human animal of the invention can be produced in accordance with well-known methods in the art. Non-limiting examples of such methods include viral integration, microinjection of zygotes and the like.

"Single Strand Conformational Polymorphism (SSCP)" refers to a method for detecting the presence of a base pair change in an amplified DNA fragment. The method involves denaturing the double stranded amplified DNA and comparing the band pattern in a known non-mutant fragment to that of an unknown fragment. A shift in the band pattern is indicative of a base pair change.

The designation "gene therapy" defines an attempt to treat disease by genetic modification of the cells of a patient.

"Allele Specific Oligonucleotide (ASO)" are designed to detect known and identified base pair change by designing oligonucleotides that are specific to the DNA fragment with and without the base change. These oligonucleotides are used as probes in hybridisation protocols under stringent conditions. Differences in the hybridization patterns is indicative of the presence or absence of the base change.

"Artificially Created Restriction Site (ACRS)" refers to a method for detection a known base change in a DNA sequence. It involves the designing of a primer that may either create or obviate a restriction site in the vicinity of known base change, such that the restriction endonuclease used can have a different digestion pattern for the changed and unchanged base.

For certainty, the sequences and polypeptides useful to practice the invention include without being limited thereto mutants, homologs, subtypes, alleles and the like. It shall be understood that generally, the sequences of the present invention should encode a functional (albeit defective) interaction domain. It will be clear to the person of ordinary skill that whether an interaction domain of the present invention, variant, derivative, or fragment thereof retains its function in binding to its partner can be readily determined by using the teachings and assays of the present invention and the general teachings of the art.

Also contemplated within the scope of the present invention are fusion proteins comprising one of the interacting domains of the present

invention. As exemplified herein below in one embodiment, at least one of an interaction domain of the present invention may be provided as a fusion protein. The design of constructs therefor and the expression and production of fusion proteins and are well known in the art (Sambrook et al., 1989, *supra*; and Ausubel et al., 1994, *supra*). Non-limiting examples of fusion proteins include LexA-fusions, B42-fusions, hemaglutinin fusions and Gluthione-S-transferase (GST) fusions and Maltose binding protein (MBP) fusions. In certain embodiments, it might be beneficial to introduce a protease cleavage site between the two polypeptide sequences which have been fused. Such protease cleavage sites between two heterologously fused polypeptides are well known in the art.

In certain embodiments, it might also be beneficial to fuse the interaction domains of the present invention to signal peptide sequences enabling a secretion of the fusion protein from the host cell. Signal peptides from diverse organisms are well known in the art. Bacterial OmpA and yeast Suc2 are two non limiting examples of proteins containing signal sequences. In certain embodiments, it might also be beneficial to introduce a linker (commonly known) between the interaction domain and the heterologous polypeptide portion. Such fusion protein find utility in the assays of the present invention as well as for purification purposes, detection purposes and the like.

The interaction domains of the present invention can be modified, for example by *in vitro* mutagenesis, to dissect the structure-function relationship thereof and permit a better design and identification of modulating compounds (i.e. to deactivate or inhibit the normal protein). However, some derivative or analogs having lost their biological function of interacting with their respective interaction partner may provide further advantages as compared to known mutant protein, as well they may also find utility, for example for raising antibodies. Such analogs or derivatives could be used for example to raise antibodies to the interaction domains of the present invention. These antibodies could be used for detection or purification purposes. In addition, these antibodies

could also act as competitive or non-competitive inhibitor and be found to be modulators of the protein-protein interactions of the present invention.

In general, techniques for preparing antibodies (including monoclonal antibodies and hybridomas) and for detecting antigens using antibodies are well known in the art (Campbell, 1984, In "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", Elsevier Science Publisher, Amsterdam, The Netherlands) and in Harlow et al., 1988 (in: Antibody - A Laboratory Manual, CSH Laboratories). The present invention also provides polyclonal, monoclonal antibodies, or humanized versions thereof, chimeric antibodies and the like which inhibit or neutralize their respective interaction domains and/or are specific thereto.

The present invention also provides antisense nucleic acid molecules which can be used for example to decrease or abrogate the expression of the normal allele. An antisense nucleic acid molecule according to the present invention refers to a molecule capable of forming a stable duplex or triplex with a portion of its targeted nucleic acid sequence (DNA or RNA). The use of antisense nucleic acid molecules and the design and modification of such molecules is well known in the art as described for example in WO 96/32966, WO 96/11266, WO 94/15646, WO 93/08845, and USP 5,593,974. Antisense nucleic acid molecules according to the present invention can be derived from the nucleic acid sequences and modified in accordance to well known methods. For example, some antisense molecules can be designed to be more resistant to degradation to increase their affinity to their targeted sequence, to affect their transport to chosen cell types or cell compartments, and/or to enhance their lipid solubility by using nucleotide analogs and/or substituting chosen chemical fragments thereof, as commonly known in the art.

From the specification and appended claims, the term therapeutic agent should be taken in a broad sense so as to also include a combination of at least two such therapeutic agents. Further, the DNA segments or proteins according to the present invention can be introduced into individuals

in a number of ways. For example, erythropoietic cells can be isolated from the afflicted individual, transformed with a DNA construct according to the invention and reintroduced to the afflicted individual in a number of ways, including intravenous injection. Alternatively, the DNA construct can be administered directly to the afflicted individual, for example, by injection in the bone marrow. The DNA construct can also be delivered through a vehicle such as a liposome, which can be designed to be targeted to a specific cell type, and engineered to be administered through different routes.

For administration to humans, the prescribing medical professional will ultimately determine the appropriate form and dosage for a given patient, and this can be expected to vary according to the chosen therapeutic regimen (i.e DNA construct, protein, cells), the response and condition of the patient as well as the severity of the disease.

Composition within the scope of the present invention should contain the active agent (i.e. fusion protein, nucleic acid, and molecule) in an amount effective to achieve the desired therapeutic effect while avoiding adverse side effects. Non-limiting examples of active agents include the mutant protein according to the present invention a nucleic acid molecule encoding such a mutant protein, an antisense molecule to a normal allele according to the present invention, etc. Typically, the nucleic acids in accordance with the present invention can be administered to mammals (i.e. humans) in doses ranging from 0.005 to 1 mg per kg of body weight per day of the mammal which is treated. Pharmaceutically acceptable preparations and salts of the active agent are within the scope of the present invention and are well known in the art (Remington's Pharmaceutical Science, 16th Ed., Mack Ed.). For the administration of polypeptides, antagonists, agonists and the like, the amount administered should be chosen so as to avoid adverse side effects. The dosage will be adapted by the clinician in accordance with conventional factors such as the extent of the disease and different parameters from the patient. Typically, 0.001 to 50 mg/kg/day will be administered to the mammal.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the invention, reference will now be made to the accompanying drawings, showing by way of illustration a preferred embodiment thereof, and in which:

Figure 1 (e-a) shows the sequence that encodes the wild-type *TIGR*/MYOC cDNA sequence (SEQ ID NO:1) localized at the *GLC1A* locus on chromosome 1q23-q25.

Figure 2 shows the characterization of a carrier homozygous for the Lys423Glu *TIGR* mutation. *a*, Structure of the *TIGR* encoded protein. The leucine zipper domain (amino acids 117-166) is shown within the N-terminal half of the protein. The Lys423Glu mutation is depicted by an open circle in the olfactomedin homology domain represented by a striped box within the C-terminal half of the protein. Amino acids comparison between human *TIGR/MYOC* protein (amino acids 415-437), human neuronal olfactomedin, rat and bullfrog neuronal olfactomedin-related proteins (GeneBank accession U79299, U03417, L13595, respectively) and C. elegans F11c3.2 protein (GeneBank accession Z81499) is represented. Identical amino acids are shaded in black, conserved amino acids are further boxed by white squares. The codon numbers correspond to those of the *TIGR/MYOC* protein, b, Identification of an homozygous carrier of the Lys423Glu *TIGR* mutation. Direct sequencing of genomic DNA revealed that persons VI-3 and VI-9 were, respectively, heterozygotic and homozygotic carriers of the Lys423Glu *TIGR* mutation. The arrows indicate the A to G transition. Person VI-2 carried two wild-type *TIGR* alleles.

Figure 3 shows the amplification refractory mutation system (ARMS) as a method to type specific alleles at a polymorphic locus. In the present invention, this method, ARMS, was used for detecting a specific pathogenic mutation. The allele-specific oligonucleotide primers were designed to discriminate between two target DNA sequences (wild-type (normal) versus pathogenic) that differed by a single nucleotide in the region of interest (either

one of the two mutations). Designed primers that differed at the extreme 3' terminus were synthetised. This was done because the DNA synthesis step in the PCR reaction is crucially dependent on correct base pairing at the 3' end. The primers that were designed are differing in their 3' ends and can therefore specifically amplify the DNA fragment of interest, either normal or mutated. This figure is a pictorial representation of ARMS for the adenine to guanine transition at nucleotide 1267. The amplification strategy is demonstrated for the wild-type, or non-mutant allele, and the mutant allele.

Figure 4 shows the phenotypic status and segregation analyses of the *GLC1A* disease haplotype and Lys423Glu in family GV-510. All living individuals were investigated for glaucoma, genotyped with microsatellite markers spanning the *GLC1A* locus and tested for the presence of the Lys423Glu *TIGR* mutation using ARMS. Selected AFM markers with their corresponding GDB number, number of alleles observed for each marker in pedigree GV-001 and sizes of the allele associated with the *GLC1A* disease haplotype are represented on top. The position of the *TIGR* gene is indicated relative to genetic markers. Sex-averaged recombination distances, depicted between marker loci in centiMorgans, were not drawn to scale. Glaucoma patients are depicted by solid black symbols, unaffected individuals by open symbols, and deceased subjects reported as blind by at least two independent family members by a black quadrant in the upper left corner of their respective symbols. OHT persons are represented by open symbols containing a central solid dot. Present ages of normal and OHT patients as well as ages of affected carriers at time of diagnosis are depicted above their respective symbols. A solid black box indicates the common *GLC1A* disease haplotype. The right side of each phased haplotype indicates the haplotype inherited from the father; the left side indicates the haplotype inherited from the mother. An asterisk in the genotype of person VII-5 represents a microsatellite mutation at locus D1S2790. Person VII-5 also inherited a paternal recombination between loci D1S2815 and D1S2790. Results of the ARMS tests are depicted below each subject's

genotype; W, ARMS test performed using the wild-type primers; M, ARMS test performed using the Lys423Glu mutant primers. The internal control PCR product is shown. Persons VI-2, VI-5, VI-6, VI-10 and VI-12 carried the wild-type allele on both chromosomes 1. Persons VI-1, VI-7, VI-9 and VI-11 are wild-type negative and mutant positive, therefore, homozygous for the Lys423Glu mutation. All other individuals are both wild-type positive and mutant positive, therefore, heterozygotes for the mutation.

Figure 5 shows the characterization of carriers for the HIS366Gln and Gln368Stop *TIGR* mutations. *a*, Structure of the *TIGR* encoded protein. The leucine zipper domain (amino acids 117-166) is shown within the N-terminal half of the protein. The His366Gln mutation is depicted by a black circle in the olfactomedin homology domain represented by a striped box within the C-terminal half of the protein. The Gln368Stop mutation is depicted by a stop codon in the olfactomedin homology domain. The codon numbers correspond to those of the *TIGR* protein. *b*, Identification of carriers for the His366Gln and Gln368Stop *TIGR* mutations. Direct sequencing of genomic DNA revealed that persons CT-003 and LA-002 were, respectively, heterozygotic carriers of the Gln368Stop and His366Gln *TIGR* mutations. The arrows indicate the C to T transition or C to G transversion.

Figure 6 shows that the TIGR wild-type protein and Lys423Glu mutated TIGR protein form high molecular weight complexes in vivo and in vitro. **a,** Western analyses of TIGR wild-type and TIGR Lys423Glu polypeptides in transfected cells. COS-7 cells were transiently transfected with expression vectors containing wild-type TIGR, pRc/CMV or TIGRK423E (pRcTIG432E) cDNA (20 mg per sample), alone or in combination. Twenty-four hours after transfection, protein extracts from total cell lysates were resolved by SDS-PAGE and TIGR polypeptides were detected by immunoblotting. The two lines above the immunoblot depict the relative amount of each cDNA tested per sample on a scale of 0 (0 mg) to 100 (20 mg). For instance, TIGR wild-type (TIGRwt) 50- TIGR Lys423Glu (TIGRK423E) 50 indicates that 10 mg of each

cDNA were added in this transfection. Two high molecular weight complexes are generated by TIGR polypeptides in COS-7 cells **b,** Western analyses of extracts obtained from dissected human trabecular meshwork (HTM) tissues. This analysis shows that TIGR polypeptides also form high molecular weight complexes in vivo. COS-7 cell extracts from cultures transfected with TIGRwt or TIGRK423E cDNA (20 mg) are depicted for comparison. Three distinct subtypes of TIGR monomers are observed after treating HTM tissue extracts with DTT. A brief exposure time period was performed to clearly visualize these monomers. **c,** Western analyses of in vitro synthesized TIGR wild-type and TIGR Lys423Glu polypeptides. pRcTIG or pRcTIG423E plasmids were added to a reticulocyte lysate transcription/translation coupled system containing pancreatic microsomal membranes. 0.5 microgram of each plasmid were tested per reaction. This analysis shows that both TIGR wild-type and TIGR Lys423Glu polypeptides can form homodimers in vitro. **a-c,** All samples were separated on 6% denaturing polyacrylamide gels and analyzed using a specific polyclonal antibody made against the non-glycosylated form of the protein. DTT indicates samples that were treated with dithiothreitol (0.1 mM) prior to electrophoresis. Position of molecular weight markers and of the expected monomers, dimers and tetramers are illustrated adjacent to the immunoblots.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following non-restrictive description of preferred embodiments with reference to the accompanying drawing which is exemplary and should not be interpreted as limiting the scope of the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The invention thus concerns novel mutations in the *TIGR* gene, a quick method for an easy detection of identified mutations and the teachings for the first time of mutant homozygotes being phenotypically normal in an autosomally dominant inherited disease.

The surprising and unexpected showing that homoallelic complementation restores a normal phenotype opens the way to new and powerful therapeutic approaches for diseases or conditions in which such a type of heterozygous state (AA') leads to a deleterious defect due to interference between the protein products of the two different alleles A and A', and in particular to an autosomal dominance state.

The present invention is illustrated in further detail by the following non-limiting examples.

EXAMPLE 1

Pedigrees and ophthalmologic assessments

1.1 Pedigree reconstitution

The pedigree genealogy was reconstituted using the registers compiled from the Catholic parish records, which systematically list births, marriages, and deaths of 98% of the Quebec population. Validation of the family tree and new data on recent births were obtained through interviews with key family members. The Archives Nationales du Quebec, the Quebec Civil register, and the Institut de recherche sur l'étude des populations (IREP) data base (Bouchard et al., 1991, Histoire d'un génome. Population et génétique dans l'est du Quebec, Presses de l'Université Laval, Sillery, Quebec, pp 607) were also consulted.

1.2 Ophthalmologic investigations

All subjects, affected or not, gave informed consent before entering the study. Clinical assessments comprised complete ophthalmologic evaluation, including best corrected visual acuity; optic disk examination; slit-lamp biomicroscopy; applanation tonometry; gonioscopy; and visual-field evaluation. Three criteria were required for primary open-angle glaucoma (POAG) diagnosis: a) intraocular pressures above 22 mm Hg in both eyes, b) characteristic optic disk damage and/or visual field impairment, and c) grade III or IV (open-angle) gonioscopy. In the absence of optic disk damage or visual-field alteration, subjects with intraocular pressures above 22 mm Hg in both eyes and grade III

or IV gonioscopy were diagnosed with ocular hypertension (OHT). Members of the families were considered normal when they presented normal optic disks and showed highest intraocular pressures ever recorded at 22 mm Hg or less. Persons with other forms of glaucomas, including grade 0 (closed angle); grade I or II (narrow-angle); congenital; and secondary glaucomas, or with other nonglaucomatous ocular disorders were considered unaffected. Blindness in deceased ancestors was confirmed by at least two independent sources.

EXAMPLE 2

Genetic analyses

2.1 Source of DNA

Blood samples were obtained from direct descendants of the founder as well as spouses of affected patients with children; from each, 20 ml of blood was drawn by venipuncture in heparinized tubes. One additional 10 ml blood sample was drawn from each subject to establish lymphoblastoid cell lines using the method of Anderson et al. (1984, In Vitro, 20:856-858).

2.2 Isolation of DNA

DNA was extracted from whole blood using the guanidine hydrochloride-proteinase K method developed by Jeanpierre (1987, Nucl. Acids. Res. 15:9611-9611).

2.3 Genotyping procedures

To accelerate genotyping, a protocol similar to the procedure of Vignal et al. (1993, Methods in molecular genetics, Academic Press, 1:211-221) which was derived from the multiplex sequencing technique of Church and Kieffer-Higgins (1988 Science 240:185-188) was used. Briefly, polymerase chain reactions (PCR) were performed in a total volume of 50 µl containing 100 ng of genomic DNA, 50 pmol of each primer, 125 mM dNTPs, 50 mM KCl, 10 mM Tris (pH 9), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X-100, and 1 U Taq polymerase (Perkin-Elmer-Cetus). Amplifications were carried out using a "hot-start" procedure. Taq polymerase was added after a 5-min denaturation step at 96°C.

Samples were then processed through 35 cycles of denaturation (94°C for 40 s) and annealing (55°C for 30 s), followed by one last step of elongation (2 min at 72°C). Usually, three amplification products synthesized with separate primer sets on identical DNA samples were coprecipitated and comigrated in a single lane of 6% polyacrylamide denaturing gels. Separated products were then transferred onto Hybond N⁺ nylon membranes (Amersham), hybridized with a $(CA)_{20}$ oligomer 3' labeled with Digoxigenin-11-ddUTP, and detected by chemiluminescence using the DIG system (Boehringer-Mannheim) with Kodak XAR-5 films. Genotypes were scored relative to reference alleles of the mother of the CEPH family 1347 (individual 134702). Genotyping was repeated upon detection of incompatibilities or recombination events.

2.4 Selection of microsatellite markers

In Figure 4, the markers used for haplotype analyses are shown. With the exception of two markers (AFMGLC21 and AFMGLC22), all AFM (Généthon) markers reported above were described in Dib et al. (1996, supra). For AFMGLC21, the sequences were primer a: GATCTCTTATCAGTCAGGCA (SEQ ID NO:7), and primer m: TTTCTAAGGCTGAATAATATTCG SEQ ID NO:8). For AFMGLC22, the sequences were primer a: TTAACTCACCACTCCCTGCC (SEQ ID NO:9), and primer m: AATTATGGCCTTCGCCC (SEQ ID NO:10). Assignment of the genetic location of these markers was established according to the method of Weissenbach et al. (1992, Nature, 359:795-801) and has been validated by construction of a 10-cM physical map (Clépet et al., 1996, Eur. J. Hum.Genet., 4:250-259).

2.5 Haplotype analysis

Haplotypes were analysed to phase the marker genotypes with the disease gene. The haplotype inherited by an affected child constituted the "disease" haplotype and was compared with the common disease haplotype inherited from the founder. The remaining three haplotypes were considered the "normal" haplotypes.

EXAMPLE 3

Discovery of the phenotypic normal-homozygote mutant

3.1 Phenotypic normal-homozygote mutant (Figure 4)

Phenotypic status and segregation analyses of the *GLC1A* disease haplotype and Lys423Glu *TIGR* mutation in family GV-510. All living individuals were investigated for glaucoma, genotyped with microsatellite markers spanning the *GLC1A* locus and tested for the presence of the Lys423Glu *TIGR* mutation using ARMS. Selected AFM markers with their corresponding GDB number, number of alleles observed for each marker in pedigree GV-001 and sizes of the allele associated with the *GLC1A* disease haplotype are represented on top. The position of the *TIGR/MYOC* gene is indicated relative to genetic markers. Sex-averaged recombination distances, depicted between marker loci in centiMorgans, were not drawn to scale. Glaucoma patients are depicted by solid black symbols, unaffected individuals by open symbols, and deceased subjects reported as blind by at least two independent family members by a black quadrant in the upper left corner of their respective symbols. OHT persons are represented by open symbols containing a central solid dot. Present ages of normal and OHT patients as well as ages of affected carriers at time of diagnosis are depicted above their respective symbols. A solid black box indicates the common *GLC1A* disease haplotype. The right side of each phased haplotype indicates the haplotype inherited from the father; the left side indicates the haplotype inherited from the mother. An asterisk in the genotype of person VII-5 represents a microsatellite mutation at locus D1S2790. Person VII-5 also inherited a paternal recombination between loci D1S2815 and D1S2790. Results of the ARMS tests are depicted below each subject's genotype; W, ARMS test performed using the wild-type primers; M, ARMS test performed using the Lys423Glu mutant primers. The internal control PCR product is shown. Persons VI-2, VI-5, VI-6, VI-10 and VI-12 carried the wild-type allele on both chromosomes 1. Persons VI-1, VI-7, VI-9 and VI-11 are wild-type negative and mutant positive, therefore, homozygous for the Lys423Glu mutation. All other

individuals are both wild-type positive and mutant positive, therefore, heterozygotes for the mutation.

3.2 Initial screening for mutations (Figure 2)

To obtain a wild-type *TIGR* cDNA, RT-PCR was performed using the Superscript RT protocol (Gibco/BRL), 500 ng of oligo-dT and 10 µg of total RNA isolated from a pool of trabecular meshwork tissue dissected from 10 pairs of human eyes. To obtain the mutated *TIGR* cDNA, the same protocol was followed using 10 µg of total RNA isolated from homozygote VI-9 immortalized lymphoblasts. One to 3 µl of first strand cDNA synthesis was amplified with primers 41F: AGAGCTTTCCAGAGGAAGCC (SEQ ID NO:11), and 1731R: GGTCTACGCCCTCAGACTAC (SEQ ID NO:12), before a second round of PCR with internal primers 31F: AGAGACAGCAGCACCCAACG (SEQ ID NO:13), and 21R: TCTGCCATTGCCTGTACAGC (SEQ ID NO:14). PCR products were directly cloned into the pCRII vector using the TA cloning kit (InVitrogen) according to the manufacturer's protocol. Cloned products were sequenced using the T7 sequencing kit (Pharmacia).

3.3 Sequencing

To confirm mutations, genomic DNA sequencing was also performed on selected individuals by direct asymmetric PCR sequencing using modifications of the protocol described by Gyllensten et al. (1988, Proc. Natl. Acad. Sci., 85:7652-7656). The mutation was recognized by the approximately equal peak intensity of the bands on the autoradiogram. All sequencing was performed bidirectionally.

EXAMPLE 4

Two mutations including ARMS

4.1 ARMS test for the Lys423Glu mutation (Figure 3)

To test for the presence of the Lys423Glu mutation, we developed an amplification refractory mutation system (ARMS) exploiting procedures described by Little (1997, Current Protocols in human genetics, Eds. Dracopoli,

N.C. et al., 9.8.1. - 9.8.12). Two complementary PCR reactions were conducted with the same substrate. The first reaction contained a forward primer specific for the wild-type allele, SEQ. NO. 3, (*GLC1A*1313AA): TCGAACAAACCTGGGAGACAAACATCCGAA. The second reaction contained a forward primer specific for the Lys423Glu *TIGR* allele, SEQ. NO. 2, (*GLC1A*1313GG): TCGAACAAACCTGGGAGACAAACATCCGGG. In each reaction, a common reverse primer, *GLC1A*1479R, was used; its sequence was: SEQ. NO. 4 CAAAGAGCTTCTTCTCCAGGGGGTTGTAGT. Both reactions gave a 225 bp amplified fragment. To serve as internal control, a second pair of primers that co-amplified a 438 bp fragment within *TIGR* exon 1 was added to the ARMS reaction. The forward *TIGR* exon 1 primer was: AGAGCTTTCCAGAGGAAGCC (SEQ ID NO:11), the reverse *TIGR* exon 1 primer was TTGGGTTTCCAGCTGGTC (SEQ ID NO:15). PCR was performed using standard protocols, annealing temperature was at 60°C. Amplification products were electrophoresed in 1,5% agarose gels before ethidium staining and scored by two independent observers.

4.2 ARMS test for the His366Gln mutation

To test for the presence of the His366Gln mutation, we developed an amplification refractory mutation system (ARMS) exploiting procedures described by Little (1997). Two complementary PCR reactions were conducted with the same substrate. The first reaction contained a forward primer specific for the wild-type allele, SEQ. NO. 6 (*GLC1A*1098CT): GAGAAGGAAATCCCTGGAGCTGGCTACCTC. The second reaction contained a forward primer specific for the His366Gln *TIGR* allele, SEQ. NO. 5, (*GLC1A*1098GT): GAGAAGGAAATCCCTGGAGCTGGCTACCTG. In each reaction, SEQ NO:4 was used as a common reverse primer. Both reactions gave a 393 bp amplified fragment. To serve as internal control, a second pair of primers that co-amplified a 438 bp fragment within *TIGR* exon 1 was added to the ARMS reaction. The forward *TIGR* exon 1 primer was: AGAGCTTTCCAGAGGAAGCC (SEQ ID NO:11), the reverse *TIGR* exon 1

primer was TTGGGTTTCCAGCTGGTC (SEQ ID NO:15). PCR was performed using standard protocols, annealing temperature was at 60°C. Amplification products were electrophoresed in 1.5% agarose gels before ethidium staining and scored by two independent observers.

EXAMPLE 5

The TIGR wild-type protein and Lys423Glu mutated TIGR protein form high molecular weight complexes in vivo and in vitro

Methods

Construction of cDNA expression vectors. To create an eukaryotic expression vector encoding the wild-type TIGR cDNA, a 1831 bp HindIII/NotI fragment encompassing 36 bp of the 5' untranslated region, the full-length 1512 bp ORF and 188 bp of the 3' untranslated region of TIGRwt cDNA was purified from pTIGwt and subcloned directionnally into the HindIII/NotI sites of plasmid pRc/CMV (Invitrogen). This new construct was named pRcTIG and produced high constitutive levels of mRNA due to the presence of CMV enhancer-promoter sequences. An expression vector encoding a TIGR cDNA carrying the Lys423Glu mutation was then generated by site-directed mutagenesis of pRcTIG using the QuikChange mutagenesis kit (Stratagene). The primers used to generate the A to G transition in pRcTIG were: 5'-GGGAGACAAACATCCGTGAGCAGTCAGTCGCC-3' (SEQ ID NO:16) and 5'-GGCGACTGACTGCTCACGGATGTTTGTCTCCC-3' (SEQ ID NO:17). This second expression vector was named pRcTIG423E. All constructions were verified by sequencing the inserts bidirectionnally between the HindIII and NotI sites.

Transfections

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin (Gibco BRL)). Cells were plated at a density of 2x106 cells per 75 cm2 tissue culture

flask. Twenty-four h later, transient transfection was performed using the DNA/DEAE-dextran transfection method coupled with a chloroquine treatment and followed by a dimethylsulfoxide shock. Each DNA/DEAE-dextran transfection sample contained a total of 20 mg/flask of plasmid pRcTIG or pRcTIG423E, alone or in combination. Briefly, COS-7 cells were exposed to the DNA/DEAE-dextran solution (0.5 mg/ml of DEAE in PBS; 5ml/flask) for 30 min before addition of 20 ml of DMEM (w/o serum)/flask containing chloroquine to reach a final concentration of 0.02 mg/ml for a further 2.5 h incubation period. The solutions were then aspirated and the cells exposed to DMSO (10% DMSO in 5 ml DMEM with serum/flask) for exactly 90 seconds. The solutions were again aspirated and the cells incubated in 25 ml DMEM supplemented with 10% fetal bovine serum and antibiotics for 2 days before protein extraction. The culture medium was replaced between the second and third day.

Reticulocyte lysate transcription-translation coupled system. In vitro transcription/translation was performed using the TNT Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's protocol. As both pRcTIG and pRcTIG423E constructs carried the T7 RNA promoter, no additional DNA modifications were done before testing. Reactions contained 0.5 microgram of each plasmid and a complete amino acid mixture instead of 35S-methionine. Canine Pancreatic Microsomal Membranes (Promega) (2.5 ml (2EQ/ml)/ 25 ml reaction) were added to the samples to optimize cotranslational processing. The samples were incubated for 90 min at 30°C before protein analysis.

Protein extraction

SDS-PAGE and Western-blot analysis. Confluent cells from tissue culture flasks were washed twice with cold PBS, scraped in 2 ml of ice cold lysis buffer (10 mM tris-HCl pH 7.5) containing the Complete Protease Inhibitor Cocktail (Boehringer Mannheim) (1 tablet/40 ml Tris-HCl). Human trabeculum meshwork (HTM) tissues were dissected from donor eyes obtained from the CHUL Eye Bank and lysed in 1 ml of cold lysis buffer. Protein

concentration was estimated and, dithiothreitol (DDT; 0.1 mM) was added to selected samples for 10 minutes, prior to electrophoresis, to reduce disulfide links that may be involved in multimerization. SDS-PAGE was performed on 6% resolving acrylamide gels using 5% stacking gels. Protein were transferred onto nitrocellulose, using a Trans-Blot SD Semi-Dry Transfer Cell apparatus (Bio-Rad) for 1 h at 150 mA. Nitrocellulose membranes were saturated for 4 h at 4°C in TBS containing 0.1% Tween-20 (TBS-T) and 5% (w/v) skimmed milk powder (blotto), rinsed once (15 min) in TBS-T and incubated O/N at 4°C with purified TIGR antibodies diluted (1/2000) in blotto (1%). After 1-15 min and 3 -5 min washes with TBS-T, the nitrocellulose membranes were incubated for 1 h at room temperature with goat-anti rabbit antibodies labeled with horseradish peroxidase (1:5000, Amersham) in blotto (1%), washed four times (1-15 min and 3-5 min) with TBS-T, revealed 2 min with Renaissance Western blot Chemiluminescence Reagent (Dupont NEN), and exposed to Reflection autoradiography films (Dupont NEN) during increasing periods of time ranging from 30 s to 5 min.

According to homoallelic complementation, the Lys423Glu mutation acts in a dominant negative fashion resulting in defective TIGR wildtype/Lys423Glu protein heteromultimers but functional TIGR Lys423Glu/Lys423Glu homopolymers. This model also demonstrates that a locus with a wild-type allele A and a mutant allele A', such that homozygosity for either allele has no phenotypic consequence, but the heterozygous state AA' leads to a deleterious defect due to interference between the protein products of the two different alleles. Homoallelic complementation further refers to affected heterozygotes in which some functional TIGR wild-type/wild-type and/or TIGR Lys423Glu/Lys423Glu homopolymers are also generated by an admixture of normal and mutant subunits, thereby explaining part of the phenotypic variability observ d at this locus.

Examination of the amino acid sequence of the TIGR gene product revealed two features potentially involved in protein-protein interactions:

1) a leucine zipper domain containing seven motifs within the N-terminal half of the polypeptide and 2) a conserved cysteine at position 433 in a region highly homologous to bullfrog olfactomedin in which the equivalent cysteine residue may promote the formation of disulfide-linked homopolymers (Figure 2).

The discovery that the TIGR wild-type protein and Lys423Glu TIGR mutated protein form high molecular weight complexes in vivo and in vitro (Figure 6) provides evidence for homoallelic complementation as the mechanism accounting for the unaffected status of TIGR Lys423Glu/Lys423Glu mutant homozygotes. As shown in Figure 5, the TIGR wild-type protein and Lys423Glu TIGR mutated protein form high molecular weight complexes in vivo and in vitro. COS-7 cells were transiently transfected with expression vectors encoding the TIGR wild-type or TIGR Lys423Glu gene products, alone or in combination, and newly synthesized proteins were analyzed by immunoblotting. In cells transfected with the TIGR wild-type cDNA construct, pRcTIG, bands of immunoreactivity were detected as two major complexes composed of TIGR polypeptides migrating at approximately 120 kD and 240 kD (Figure 6 a and b). When the denaturing agent dithiothreitol (DDT) was added to the extracts prior to electrophoresis, both complexes were resolved into two lower molecular weight forms migrating at approximately 57 kD and 62 kD (Figure 6 a and b). As deduced from the nucleotide sequence, the 57 kD form corresponded to TIGR monomers originating at the first translation initiation site of the TIGR mRNA while the 62 kD form represented partially glycosylated monomers. In cells transfected with increasing concentrations of TIGR Lys423Glu cDNA in the presence of decreasing amount of the wild-type construct, the pattern of immunoreactivity was identical to that detected when transfections were performed with pRcTIG alone indicating that TIGR Lys423Glu gene products did not disrupt the formation of the two major complexes (Figure 6 a and b). Immunoblotting of protein extracts obtained from human trabecular meshwork (HTM) tissues dissected from donor ey s revealed a similar pattern of migration establishing that TIGR polypeptides also formed high molecular weight

complexes in vivo (Figure 6b). Under denaturing condition, HTM TIGR polypeptides were resolved into three distinct monomers: the 57 and 62 kD products and a smaller, approximately 55 kD, TIGR polypeptide resulting from internal cleavage or, utilization of a second initiation site for translation (Figure 6b).

To show that TIGR polypeptides did not required extraneous proteins to undergo oligomerization, we used an in vitro reticulocyte lysate system in which transcription of TIGR wild-type and TIGR Lys423Glu cDNAs was coupled to translation of their respective mRNAs. Following protein extraction and immunoblotting, we observed that both TIGR wild-type and TIGR Lys423Glu polypeptides formed homodimers migrating at the expected 120 kD position (Figure 6c). These data showed that the high molecular weight complexes observed in COS-7 cells were produced by multimerization of TIGR monomers, generating homodimers and homotetramers.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, it can be modified, without departing from the spirit and nature of the subject invention as defined in the appended claims.

WHAT IS CLAIMED IS:

1. An isolated DNA comprising the nucleotide sequence defined in SEQ. ID. NO.: 1, wherein the nucleotide located at position 1267 is a guanidine residue in lieu of an adenine residue , said guanidine residue being a specific nucleotide of a mutant allele of the *TIGR* gene.

2. An isolated DNA comprising the nucleotide sequence defined in SEQ. ID. NO.: 1, wherein the nucleotide located at position 1096 is a guanidine residue in lieu of a cytosine residue , said guanidine residue being a specific nucleotide of a mutant allele of the *TIGR* gene.

3. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with said specific nucleotide , as defined in claim 1, or a complementary sequence thereof.

4. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with a non mutant nucleotide corresponding to said specific nucleotide, as defined in claim 1, or a complementary sequence thereof.

5. An oligonucleotide as defined in claim 3, which is incapable of priming a polymerase priming extension when annealed to a non mutant allele.

6. An oligonucleotide as defined in claim 4, which is incapable of priming a polymerase priming extension when annealed to said mutant allele.

7. An oligonucleotide as defined in claim 5, which has the nucleotide sequence of SEQ. ID. NO.: 2.

8. An oligonucleotide as defined in claim 6, which has the nucleotide sequence of SEQ. ID. NO.: 3.

9. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide having a nucleotide sequence shared by said mutant allele and a non mutant allele, as defined in claim 1, and a complementary sequence thereof.

10. An oligonucleotide as defined in claim 9, which has the nucleotide sequence of SEQ. ID. NO.: 4.

11. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with said specific nucleotide , as defined in claim 2, or a complementary sequence thereof.

12. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide ending at its 3' end with a non mutant nucleotide corresponding to said specific nucleotide, as defined in claim 2, or a complementary sequence thereof.

13. An oligonucleotide as defined in claim 11, which is incapable of priming a polymerase priming extension when annealed to a non mutant allele.

14. An oligonucleotide as defined in claim 12, which is incapable of priming a polymerase priming extension when annealed to said mutant allele.

15. An oligonucleotide as defined in claim 13 ,which has the nucleotide sequence of SEQ. ID. NO.: 5.

16. An oligonucleotide as defined in claim 14, which has the nucleotide sequence of SEQ. ID. NO.: 6.

17. An oligonucleotide comprising at least 10 nucleotides of SEQ. ID. NO.: 1, said oligonucleotide having a nucleotide sequence shared by said mutant allele and a non mutant allele, as defined in claim 2, and a complementary sequence thereof.

18. An oligonucleotide as defined in claim 17, which has the nucleotide sequence of SEQ. ID. NO.: 4.

19. A method for detecting a mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual with an oligonucleotide as defined in claims 3, 5 or 7 and with an oligonucleotide as defined in claim 9 or 10; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said mutant allele.

20. A method for detecting a mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide as defined in claims 11,13 or 15 and with an

oligonucleotide as defined in claim 17 or 18; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said mutant allele.

21. A method for detecting a non-mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide as defined in claim 4, 6 or 8 and with an oligonucleotide as defined in claim 9 or 10; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said non-mutant allele.

22. A method for detecting a non-mutant allele of the *TIGR* gene which comprises the steps of contacting a DNA sample taken from an individual, with an oligonucleotide as defined in claim 12, 14 or 16 and with an oligonucleotide as defined in claim 17 or 18; obtaining an amplified product in an amplification reaction; and detecting said amplification product as an indication of the presence of said non-mutant allele.

23. A kit for the detection of mutations in the *TIGR* gene comprising an oligonucleotide as defined in any one of claims 3, 5, 7, 11,13 and 15; an oligonucleotide as defined in any one of claims 4, 6, 8, 12, 14 and 16; and an oligonucleotide as defined in any one of claims 9, 10, 17 and 18; and suitable reagents required for obtaining amplified products in an amplification reaction.

24. The kit of claim 23, wherein amplification products are detectable.

25. A method for detecting in an individual the inheritance of two of said mutant alleles as defined in claim 1, said individual being homozygote for said mutant allele and phenotypically normal, and said individual being capable of transmitting said mutant allele to an offspring whereby said offspring is at risk for developing glaucoma, with said method comprising the steps of reproducing the methods of claims 19 and 21; a positive result obtained from the method of claim 19 and a negative result from the method of claim 21, being an indication that said individual is homozygote for said mutant allele.

26. A method to counteract glaucoma in a heterozygotic carrier of TIGR mutations, an overexpression of mutated TIGR protein in a patient to counteract the glaucoma phenotype associated with said heterozygocity, thus rendering the phenotype of said patient normal by homoallelic complementation.

27. A method to counteract and/or treat heterozygotic carriers of an autosomal dominant inherited disorder caused by a protein that forms homomultimers, comprising at least one of an overexpression of the mutated protein and an inhibition of the normal protein in a patient, to counteract the affected phenotype, thereby rendering said phenotype normal by homoallelic complementation.

28. The method of claim 27, wherein said autosomal dominant inherited disorder is glaucoma and said normal protein is normal TIGR protein.

29. A method to counteract glaucoma in a heterozygotic carrier of TIGR mutations comprising an inhibition of xpression of the normal

Wild-Type GLC1A/TIGR cDNA sequence

```
                    -36                                  -10
                    agagct ttccagagga agcctcacca agcctctgca

+1
ATG AGG TTC TTC TGT GCA CGT TGC TGC AGC TTT GGG CCT GAG ATG
met arg phe phe cys ala arg cys cys ser phe gly pro glu met
+1

CCA GCT GTC CAG CTG CTG CTT CTG GCC TGC CTG GTG TGG GAT GTG
pro ala val gln leu leu leu leu ala cys leu val trp asp val

91
GGG GCC AGG ACA GCT CAG CTC AGG AAG GCC AAT GAC CAG AGT GGC
gly ala arg thr ala gln leu arg lys ala asn asp gln ser gly
31
```

Fig. 1A

```
CGA TGC CAG TAT ACC TTC AGT GTG GCC AGT CCC AAT GAA TCC AGC
arg cys gln tyr thr phe ser val ala ser pro asn glu ser ser

181
TGC CCA GAG CAG AGC CAG GCC ATG TCA GTC ATC CAT AAC TTA CAG
cys pro glu gln ser gln ala met ser val ile his asn leu gln
61

AGA GAC AGC AGC ACC CAA CGC TTA GAC CTG GAG GCC ACC AAA GCT
arg asp ser ser thr gln arg leu asp leu glu ala thr lys ala

271
CGA CTC AGC TCC CTG GAG AGC CTC CTC CAC CAA TTG ACC TTG GAC
arg leu ser ser leu glu ser leu leu his gln leu thr leu asp
91

CAG GCT GCC AGG CCC CAG GAG ACC CAG GAG GGG CTG CAG AGG GAG
gln ala ala arg pro gln glu thr gln glu gly leu gln arg glu
```


Fig - 1B

361
CTG GGC ACC CTG AGG CGG GAG CGG GAC CAG CTG GAA ACC CAA ACC
leu gly thr leu arg arg glu arg asp gln leu glu thr gln thr
121

AGA GAG TTG GAG ACT GCC TAC AGC AAC CTC CTC CGA GAC AAG TCA
arg glu leu glu thr ala tyr ser asn leu leu arg asp lys ser

451
GTT CTG GAG GAA GAG AAG AAG CGA CTA AGG CAA GAA AAT GAG AAT
val leu glu glu glu lys lys arg leu arg gln glu asn glu asn
151

CTG GCC AGG AGG TTG GAA AGC AGC AGC CAG GAG GTA GCA AGG CTG
leu ala arg arg leu glu ser ser ser gln glu val ala arg leu

Fig. 1C

```
541
AGA AGG GGC CAG TGT CCC CAG ACC CGA GAC ACT GCT CGG GCT GTG
arg arg gly gln cys pro gln thr arg asp thr ala arg ala val
181

CCA CCA GGC TCC AGA GAA GTT TCT ACG TGG AAT TTG GAC ACT TTG
pro pro gly ser arg glu val ser thr trp asn leu asp thr leu

631
GCC TTC CAG GAA CTG AAG TCC GAG CTA ACT GAA GTT CCT GCT TCC
ala phe gln glu leu lys ser glu leu thr glu val pro ala ser
211

CGA ATT TTG AAG GAG AGC CCA TCT GGC TAT CTC AGG AGT GGA GAG
arg ile leu lys glu ser pro ser gly tyr leu arg ser gly glu
```

FIG - 10

721

GGA GAC ACC GGA TGT GGA GAA CTA GTT TGG GTA GGA GAG CCT CTC
gly asp thr gly cys gly glu leu val trp val gly glu pro leu
241

ACG CTG AGA ACA GCA GAA ACA ATT ACT GGC AAG TAT GGT GTG TGG
thr leu arg thr ala glu thr ile thr gly lys tyr gly val trp

811

ATG CGA GAC CCC AAG CCC ACC TAC CCC TAC ACC CAG GAG ACC ACG
met arg asp pro lys pro thr tyr pro tyr thr gln glu thr thr
271

TGG AGA ATC GAC ACA GTT GGC ACG GAT GTC CGC CAG GTT TTT GAG
trp arg ile asp thr val gly thr asp val arg gln val phe glu

Fig - 1E

```
901
TAT GAC CTC ATC AGC CAG TTT ATG CAG GGC TAC CCT TCT AAG GTT
tyr asp leu ile ser gln phe met gln gly tyr pro ser lys val
301

CAC ATA CTG CCT AGG CCA CTG GAA AGC ACG GGT GCT GTG GTG TAC
his ile leu pro arg pro leu glu ser thr gly ala val val tyr

991
TCG GGG AGC CTC TAT TTC CAG GGC GCT GAG TCC AGA ACT GTC ATA
ser gly ser leu tyr phe gln gly ala glu ser arg thr val ile
331

AGA TAT GAG CTG AAT ACC GAG ACA GTG AAG GCT GAG AAG GAA ATC
arg tyr glu leu asn thr glu thr val lys ala glu lys glu ile
```

Fig - 1F

```
1081
CCT GGA GCT GGC TAC CAC GGA CAG TTC CCG TAT TCT TGG GGT GGC
pro gly ala gly tyr his gly gln phe pro tyr ser trp gly gly
361                 366

TAC ACG GAC ATT GAC TTG GCT GTG GAT GAA GCA GGC CTC TGG GTC
tyr thr asp ile asp leu ala val asp glu ala gly leu trp val

1171
ATT TAC AGC ACC GAT GAG GCC AAA GGT GCC ATT GTC CTC TCC AAA
ile tyr ser thr asp glu ala lys gly ala ile val leu ser lys
391

CTG AAC CCA GAG AAT CTG GAA CTC GAA CAA ACC TGG GAG ACA AAC
leu asn pro glu asn leu glu leu glu gln thr trp glu thr asn
```

FIG - 1G

```
1261
ATC CGT AAG CAG TCA GTC GCC AAT GCC TTC ATC ATC TGT GGC ACC
ile arg lys gln ser val ala asn ala phe ile ile cys gly thr
421     423

TTG TAC ACC GTC AGC AGC TAC ACC TCA GCA GAT GCT ACC GTC AAC
leu tyr thr val ser ser tyr thr ser ala asp ala thr val asn

1351
TTT GCT TAT GAC ACA GGC ACA GGT ATC AGC AAG ACC CTG ACC ATC
phe ala tyr asp thr gly thr gly ile ser lys thr leu thr ile
451

CCA TTC AAG AAC CGC TAT AAG TAC AGC AGC ATG ATT GAC TAC AAC
pro phe lys asn arg tyr lys tyr ser ser met ile asp tyr asn
```

Fig - 1H

1441
CCC CTG GAG AAG AAG CTC TTT GCC TGG GAC AAC TTG AAC ATG GTC
pro leu glu lys lys leu phe ala trp asp asn leu asn met val
481

1486
ACT TAT GAC ATC AAG CTC TCC AAG ATG TGA aaagcctcc aagctgtaca
thr tyr asp ile lys leu ser lys met STOP
496

1535
ggcaatggca gaaggagatg ctcagggctc ctgggggggag caggctgaag

1585
ggagagccag ccagccaggg cccaggcagc tttgactgct ttccaagttt

1635
tcattaatcc agaaggatga acatggtcac catctaacta ttcaggaatt

Fig. 11

1685
gtagtctgag ggcgtagacc atttcatata ataaatatcc tttatcttct

1735
gtcagcattt atgggatgtt taatgacata gttcaagttt ttcctgaaaa

1785
ccattgctct tgcatgttac atggttacca caagccacaa taaaaagcat

1835
aacttctaaa ggaagcagaa

Fig. 1J

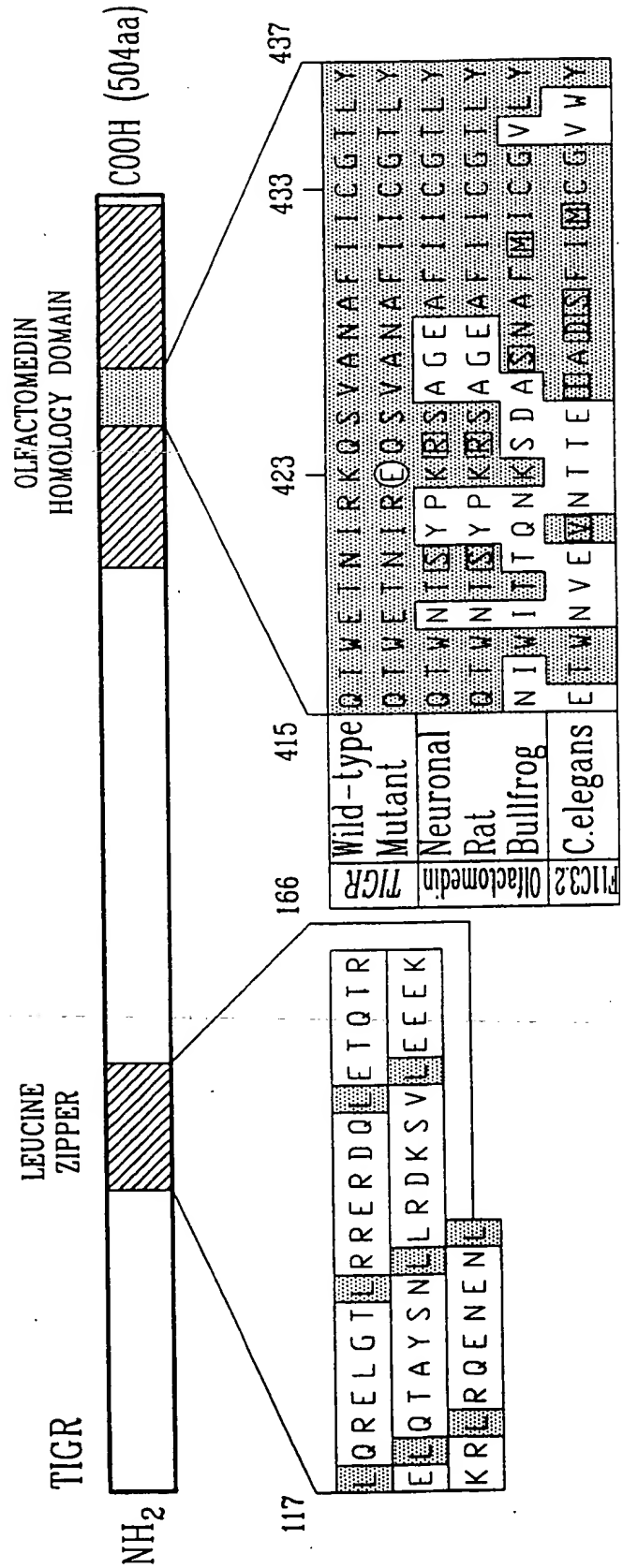

NH2
TIGR
LEUCINE ZIPPER
OLFACTOMEDIN HOMOLOGY DOMAIN
COOH (504aa)
117
166
415
423
433
437
TIGR
Wild-type
Mutant
Olfactomedin
Neuronal
Rat
Bullfrog
FLJ3.2
C.elegans

FIG - 2A

Codon	Wild-type / Mutant 3'	
425	Ser	A C T
424	Gln	G A C
423	Lys/Glu	G A A/G
422	Arg	T G C
421	Ile	C T A
		5'

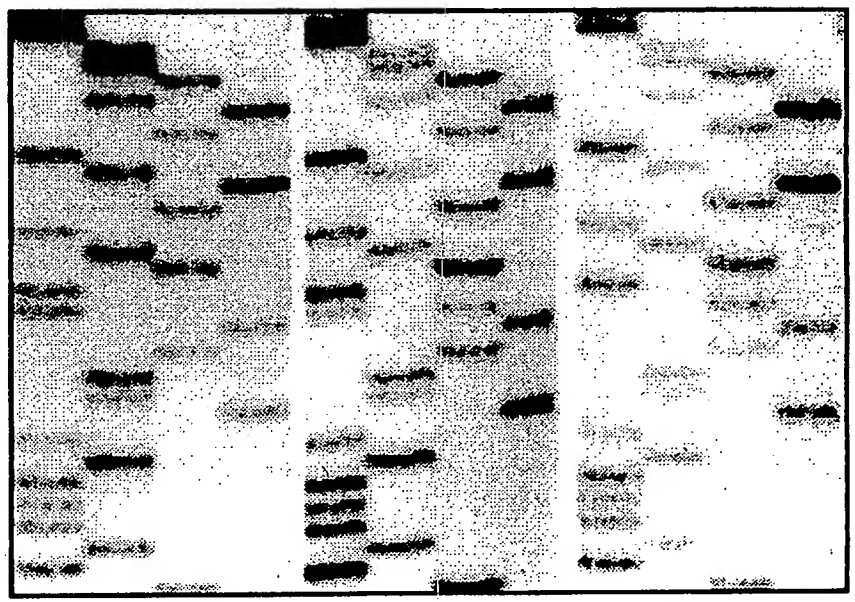


Fig. 2B

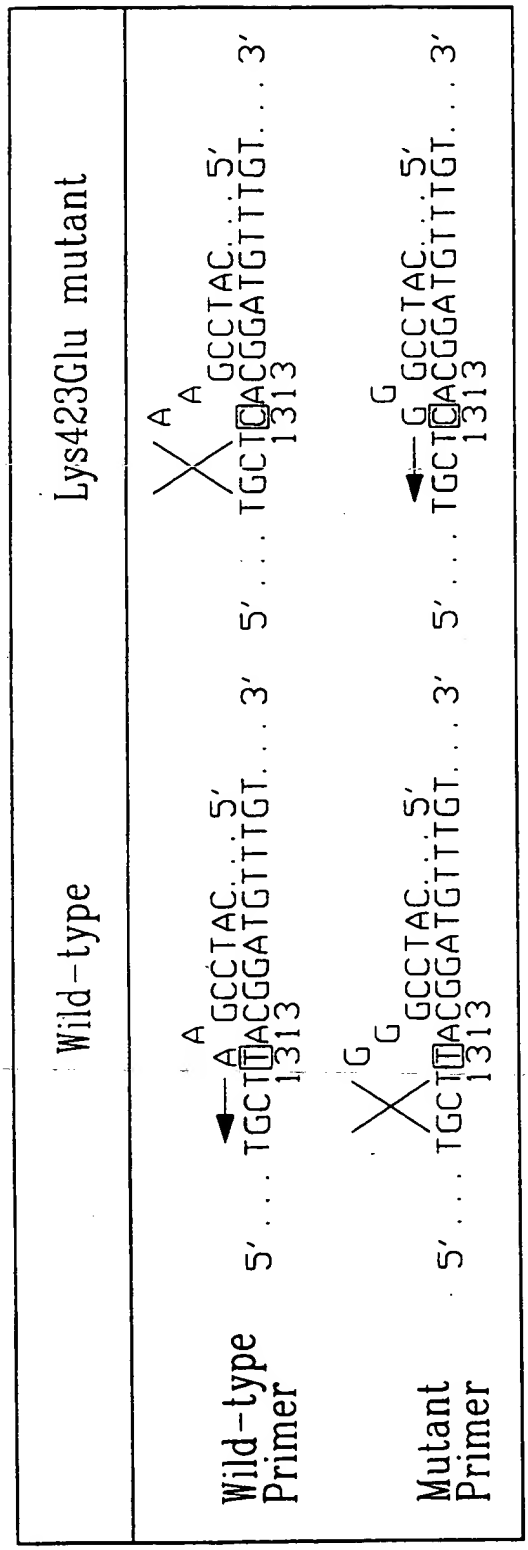

Wild-type
Lys423Glu mutant
Wild-type Primer
Mutant Primer
5'....TGCTCACGGATGTTTGT...3'
3'...ACGAGTGCCTAC...5'
1313

FIG - 3A

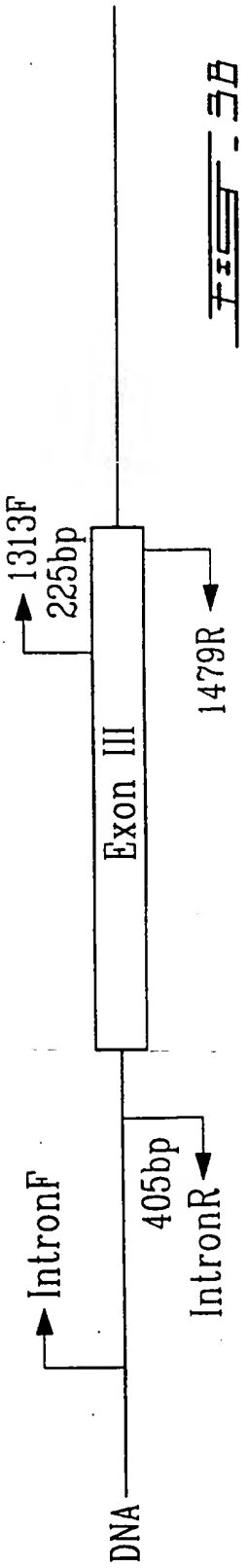

DNA
IntronF
405bp
IntronR
Exon III
1313F
225bp
1479R

FIG - 3B

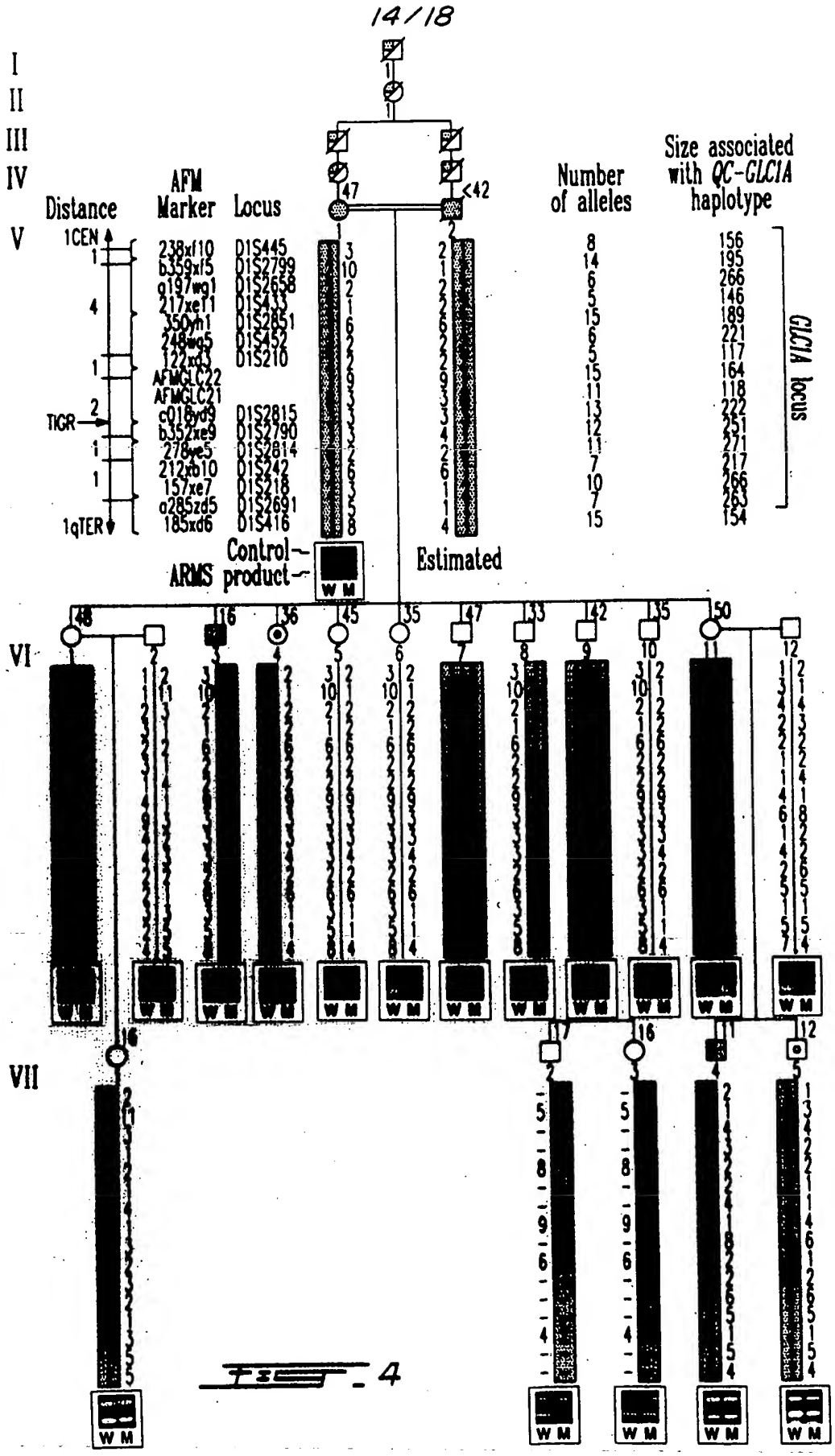

I
II
III
IV
V
VI
VII
Distance
AFM Marker
Locus
Number of alleles
Size associated with QC-GLCIA haplotype
GLCIA locus
1CEN
TIGR
1qTER
238xf10 D1S445
b359xf5 D1S2799
q197wg1 D1S2658
217xe11 D1S433
350yh1 D1S2851
248wg5 D1S452
122xd3 D1S210
AFMGLC22
AFMGLC21
c018yd9 D1S2815
b352xe9 D1S2790
278ye5 D1S2814
212xb10 D1S242
157xe7 D1S218
a285zd5 D1S2691
185xd6 D1S416
47
42
8 14 6 5 15 6 5 15 11 13 12 11 7 10 7 15
156 195 266 146 189 221 117 164 118 222 251 271 217 266 263 154
Control product
ARMS
Estimated
W M

Fig. 4

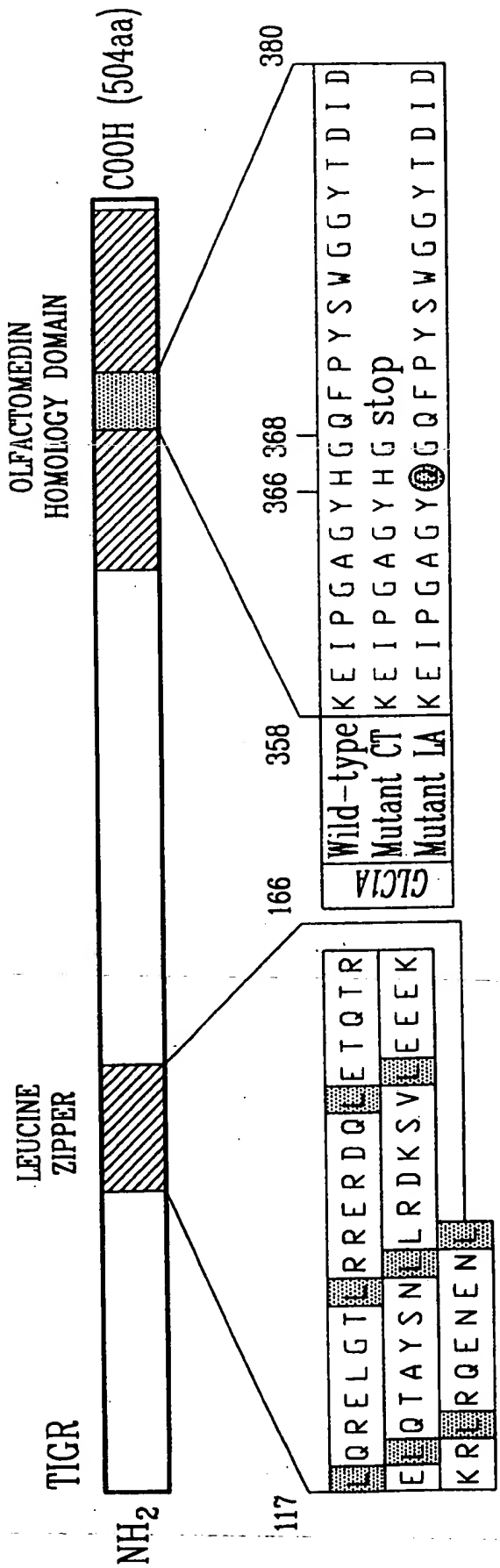
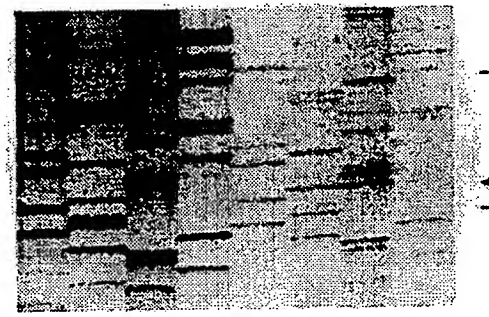

NH2
TIGR
LEUCINE ZIPPER
OLFACTOMEDIN HOMOLOGY DOMAIN
COOH (504aa)
117
166
358
366 368
380
CLCIA
Wild-type
Mutant CT
Mutant LA
KEIPGAGYHGQFPYSWGGYTDID
KEIPGAGYHG stop
KEIPGAGY@GQFPYSWGGYTDID
LQRELGTLRRERDQLETQTR
EIQTAYSNLLRDKSVLEEEK
KRLRQENENL

Fig. 5A

Codon	Wild Type / Mutant	3'
370	Pro	G C C
369	Phe	C T T
368	Gln / Stop	G A C/T
367	Gly	A G G
366	His	C A C
		5'

Person CF-003	Person LA-002
A C G T	A C G T



Codon	Wild Type / Mutant	3'
370	Pro	G C C
369	Phe	C T T
368	Gln	G A C
367	Gly	A G G
366	His/Gln	C/G A C
		5'

Fig. 5B

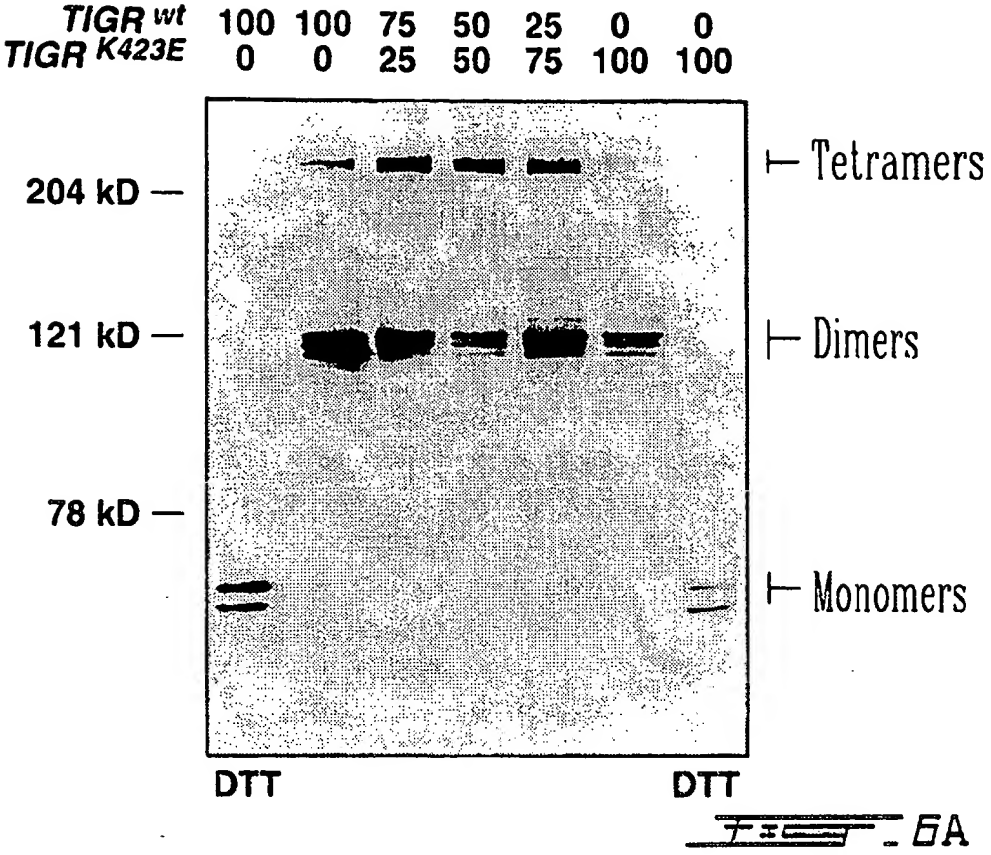

TIGR wt 100 100 75 50 25 0 0
TIGR K423E 0 0 25 50 75 100 100
204 kD
121 kD
78 kD
Tetramers
Dimers
Monomers
DTT
DTT

Fig. 6A

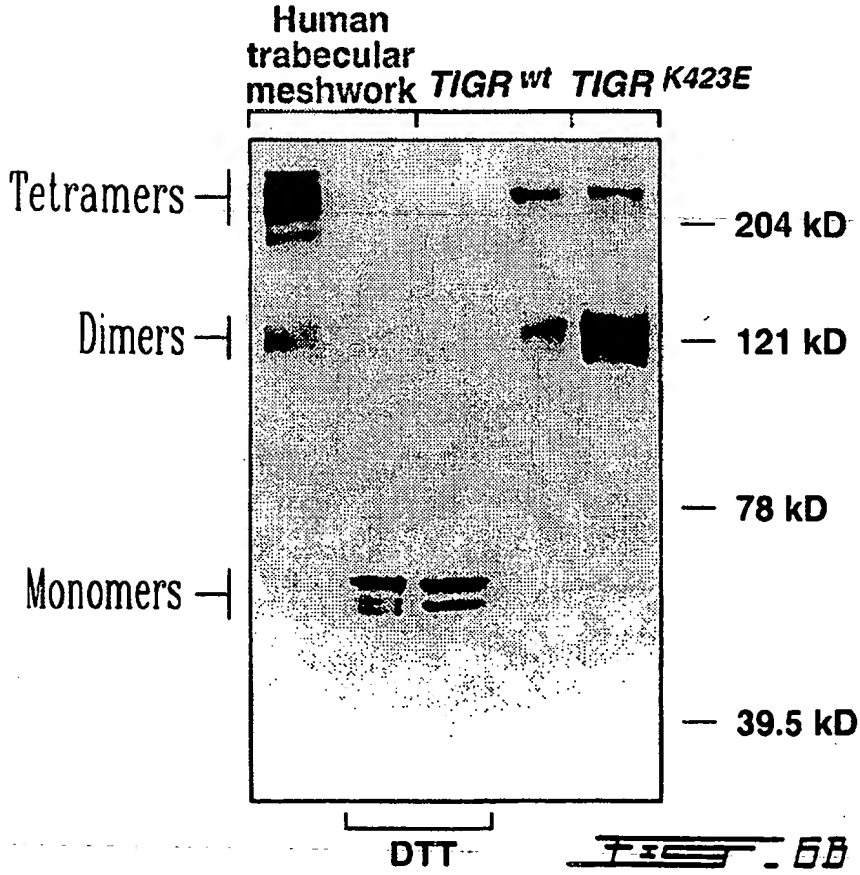

Human trabecular meshwork
TIGR wt
TIGR K423E
Tetramers
Dimers
Monomers
204 kD
121 kD
78 kD
39.5 kD
DTT

Fig. 6B

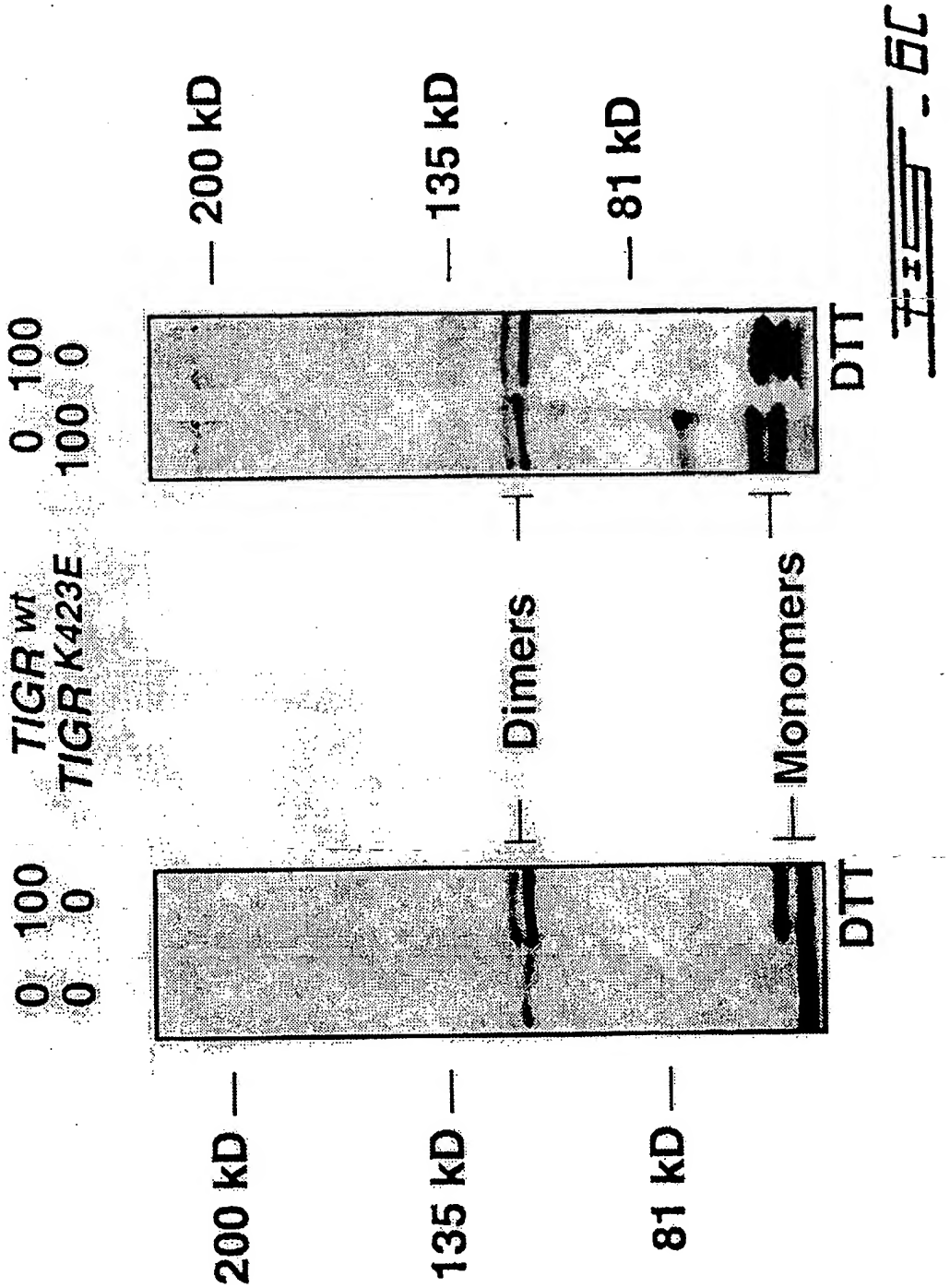

200 kD
135 kD
81 kD
0 100
100 0
DTT
TIGR wt
TIGR K423E
Dimers
Monomers
0 100
0 0
DTT
200 kD
135 kD
81 kD

Fig. 6C

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/CA 98/00923

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	RAYMOND V ET AL: "Normal homozygotes for primary open-angle glaucoma caused by the TIGR/GLCIA gene: evidence for a new form of dominance in man" AMERICAN JOURNAL OF HUMAN GENETICS, vol. 61, no. 4 (suppl), 28 October 1997 - 1 November 1997, page A21 XP002094309 see abstract 101 ---	1
Y	RAYMOND V ET AL: "Homozygotes for autosomal dominant open-angle glaucoma at the GLCIA locus" AMERICAN JOURNAL OF HUMAN GENETIC, vol. 59, no. 4 (suppl), 29 October 1996 - 2 November 1996, page A280 XP002094310 see abstract 1625 ---	1,2, 19-29
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 1999	12/03/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/CA 98/00923

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RAYMOND V ET AL: "Normal homozygotes for autosomal dominant open-angle glaucoma at tzhe GLAIA locus" INVESTIGATIVE OPTHALMOLOGY AND VISUAL SCIENCE, vol. 38, no. 4 (part 1-2), 11 - 16 May 1997, page S576 XP002094311 see abstract 2862-B327 ---	1,2, 19-29
Y	STOILOVA D ET AL: "Identification of a new TIGR mutation in a family with juvinile-onset primary open angle glaucoma" OPHTHALMIC GENETICS, vol. 18, no. 3, September 1997, pages 109-18, XP002094312 see the whole document ---	1,2, 19-29
Y	ALWARD W ET AL: "The phenotype of primary open angle glaucoma patients with mutations in the GLAIA gene" INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, vol. 38 , no. 4 (Part 1-2), 11 - 16 May 1997, page S930 XP002094313 see abstract 4335 ---	1,2, 19-29
Y	WO 96 14411 A (UNIV CALIFORNIA) 17 May 1996 see the whole document ---	1,2, 19-29
A	NEWTON C R ET AL: "ANALYSIS OF ANY POINT MUTATION IN DNA. THE AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS)" NUCLEIC ACIDS RESEARCH, vol. 17, no. 7, 11 April 1989, pages 2503-2516, XP000141596 see the whole document -----	3-18

INTERNATIONAL SEARCH REPORT

..ternational application No.
PCT/CA 98/ 00923

Box I Observati ns where certain claims were found unsearchable (Continuati n f item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 26-29 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.